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Summary

Begin the article with a short summary.

Text

Submit an original and three copies of the manuscript, typed double spaced. Each page should begin with a new paragraph. Use accepted spelling, abbreviation, and hyphenation found in *Webster's New International Dictionary*. Footnotes should be typed at the bottom of the page on which they appear.

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Proceedings of the

National Prostatic Cancer Workshop

Buffalo, New York

June 28–29, 1974

The National Prostatic Cancer Project is an organ site program of planned and coordinated research supported by the Division of Cancer Research Resources and Centers of the National Cancer Institute. This work is supported in part by USPHS grants CA-16270 and CA-14716. The planning effort began on May 1, 1972. The operational grant started on April 1, 1973. 1974 represents our first full federal fiscal year.

The objectives of this Workshop are:

a. To exchange information between investigators from different disciplines but with a common denominator of allied interests.

b. To generate ideas for participants and Working Cadre members of the National Prostatic Cancer Project in terms of direction to be taken in current and proposed research areas.

c. To publicize the program, stimulate interest in prostate physiology, and provide a background of information to new investigators in the field.

d. The Workshop will serve to coordinate the scientific activities of all grantees under this program.

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The Secretary of Health, Education, and Welfare has determined that the publication of this periodical is necessary in the transaction of the public business required by law of this Department. Use of funds for printing this periodical has been approved by the Director of the Office of Management and Budget through November 30, 1976.

Introduction

The National Prostatic Cancer Program is an extramural function of the National Cancer Institute Organ Site Programs. Although the planning effort for this endeavor, in a comprehensive fashion, began in only the spring of 1972, the results of the present workshop will indicate that a great deal has been operationally achieved. We have completed only 1 full year of the grant aspect of the program. Many additional improvements in the exchange of information, on both national and international levels, have been achieved.

The principle of peer review has been maintained. We are deeply indebted to well over 70 scientists and clinicians throughout the United States who have generously served on an ad hoc basis to assist in providing review for the numerous letters of intent and grants that the program has thus far generated. The year 1974 represents our first full federal fiscal year. Despite these understandable limitations, I believe you will see from the quality and content of the material presented at this workshop that a number of very important and timely endeavors in the etiology, prevention, detection, diagnosis, and treatment of prostatic cancer have been successfully undertaken and, in some instances, are near completion.

An important byproduct of this program has been the opportunity for coordination and exchange of ideas between other clinical cooperative groups and between working scientists throughout the United States. The focusing of interest nationally on the important problem of prostatic cancer has thus, in my opinion, been initially achieved. With such a cooperative effort and a comprehensive approach, a significant impact on our ultimate goal of reduction of morbidity and mortality of prostatic cancer will doubtless be achieved in a reasonable time.

Gerald P. Murphy

Project Director
National Prostatic Cancer Project
[A National Cancer Institute (Division of Cancer Research Resources and Centers) grant-supported program of research]
Buffalo, NY

Characterization of Prostatic Carcinoma Among Blacks: A Preliminary Report [1]

M. A. Jackson,[2] B. S. Ahluwalia,[2] E. B. Attah,[3] C. A. Connolly,[2] J. Herson,[2] M. Y. Heshmat,[2] A. G. Jackson,[2] G. W. Jones,[2] S. K. Kapoor,[2] J. Kennedy,[2] J. Kovi,[2] A. O. Lucas,[3] E. O. Nkpoeong,[3] E. Olisa,[2] and A. O. Williams [3,4]

SUMMARY

This study was designed to compare United States (Washington, DC) black prostatic carcinoma patients (high-risk group) with Nigerian (Ibadan) black prostatic carcinoma patients (low-risk group). Although the material is meager, preliminary analyses suggest that carcinoma of the prostate is a common disease in both US black men (196 of 1000 autopsies) and in Nigerian black men (67 of 1000 autopsies). The tumor tends to be of a higher histologic grade (less well differentiated) and the carcinomatous foci are more numerous in the Nigerian patients. Fifty-three percent of US patients are in stages I and II when the disease is first discovered. Plasma testosterone, estrone, and estradiol concentrations did not differ significantly between US patients and controls. A statistically significant positive association is indicated between carcinoma of the prostate and the following epidemiologic variables: racial admixture, age of puberty, and age of first coitus. The median age of necropsy cases with carcinoma was 50.0 years in Nigeria and 68.3 years in the US.

[Cancer Chemother Rep 59:3–15, 1975]

Carcinoma of the prostate gland is the leading cancer in black men in the United States, with a relative ratio frequency of approximately 20% and an annual incidence rate that reaches as high as 950 per 100,000 men in certain age groups (1). The overall incidence rate reported for this type of cancer in Alameda County, California, was 38 per 100,000 men for whites and 65.3 per 100,000 men for blacks (2). Deaths in the US from carcinoma of the prostate increased by 137% and 39% among the black and white populations respectively over the period of 1949–1967 (3). In Washington, DC, the age-standardized incidence rate (world standard) for blacks was 59.8 per 100,000 men, while in Ibadan, Nigeria, the corresponding incidence rate was 9.7 per 100,000 men (1). Because the ancestors of the US black came primarily from West Africa, the difference in incidence rates between US and Nigerian blacks suggests that an environmental factor or factors contribute to the high incidence of prostatic carcinoma in the US black male population.

Clinically silent incidental carcinoma was found in 26% of the autopsies of adult male patients in the US (white and black) who died

[1] Supported by Public Health Service grant CA-15448 from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

[2] Howard University College of Medicine, Washington, DC.

[3] University of Ibadan, University College Hospital, Nigeria.

[4] *Reprint requests to:* Marvin A. Jackson, MD, Howard University College of Medicine, Washington, DC 20059.

from nongenitourinary diseases. Autopsies of patients over 90 years of age showed incidence figures between 40% and 80% (4). No known study in US blacks exists which ascertains whether the high rate of invasive prostatic carcinoma is preceded by a similarly high rate of incidental carcinoma of the prostate. Only a few studies have been done on carcinoma of the prostate in Africa. In a limited study of prostate glands from 96 African patients (89 of them from Uganda), there was definite evidence of incidental carcinoma in ten patients and possible evidence in an additional eight patients (5). In contrast, the incidence rate for invasive carcinoma in Ugandan men was 4.4 per 100,000 men (6).

A comparison of carcinoma of the prostate gland in a high-risk US (Washington, DC) black population and in a low-risk largely indigenous Nigerian (Ibadan) black population is the purpose of this study. Specifically, the study will (*a*) compare the clinical features, plasma hormone (testosterone, estrone, estradiol, and prolactin) levels, epidemiologic factors, and tumor morphology in high-risk US blacks and low-risk African blacks; (*b*) search for factors that may be associated with the development of prostate cancer in the US and Nigeria; (*c*) determine in postmortem material the incidence of incidental (non-invasive) carcinoma of the prostate in the two population groups; and (*d*) study the relation between incidental carcinoma and non-neoplastic prostatic diseases and morphologic changes in the liver, adrenal glands, breast, testes, and pituitary gland.

MATERIALS AND METHODS

Patients

All new untreated patients with carcinoma of the prostate gland were admitted to the study. Blood samples for hormone and phosphatase analyses were drawn from all male patients after admission to the Urologic Services of Howard University Hospital, Washington, DC, DC General Hospital, Washington, DC, and University Hospital, Ibadan, Nigeria. The blood samples were collected under constant fasting premedication conditions at 8:00 AM and before examination of the prostate gland. Plasma and serum samples were frozen and stored until a histologic diagnosis of carcinoma of the prostate was established. Total alkaline (7) and acid and prostatic acid (8,9) phosphatase activities were determined from the serum samples. The plasma sample was analyzed for testosterone, estrone, and estradiol (10,11). Blood samples taken from patients without carcinoma of the prostate were discarded. Patients with histologically confirmed prostatic carcinoma were subjected to a detailed clinical evaluation and staged according to the Veterans' Administration Cooperative Urological Research Group system (12).

Epidemiology

All patients with histologically confirmed carcinoma of the prostate and all control patients were interviewed using pretested epidemiologic questionnaire forms (appendix 1). The following areas were covered in the questionnaire: social and demographic factors, family history, nutritional food habits, sexual history, past history of illnesses and operations, history of urogenital diseases, and use of contraceptives by sexual partners. Hospital controls were chosen for this study.

Control patients, age-matched with prostatic carcinoma patients, were free of clinically demonstrable neoplastic, urologic, and endocrine conditions, had not received hormonal therapy within the past 6 months, and had not received blood transfusions within 3 days preceding the collection of blood samples for hormonal and enzyme analyses.

Morbid Anatomy

The whole prostate gland was collected from consecutive necropsy cases of black men in Washington, DC, and Ibadan, Nigeria. Whole glands with attached seminal vesicles were accessioned, entered into a blind study, weighed, step-sectioned at 4-mm intervals, processed, mounted on large slides, and stained with hematoxylin and eosin (H & E) (13). In addition to the prostate glands, tissue blocks of liver, adrenal gland, testes and pituitary gland, and breast were collected from each case, processed, mounted, and stained with H & E. Each case was analyzed for specific morphologic changes according to the protocol.

From patients with the clinical diagnosis of carcinoma undergoing prostatic surgery, sections from all tissue blocks were studied. The histologic grade of the tumor and other morphologic parameters were correlated with the clinical staging, hormonal studies, and epidemiologic data. All tissue specimens for this study were evaluated in one laboratory.

RESULTS AND DISCUSSION

Nine Nigerian patients with histologically confirmed carcinoma of the prostate, 30 Nigerian necropsy cases, 33 US patients with prostatic carcinoma, 21 US control patients, and 97 US necropsy cases have been entered into the study over the past 8 months. Complete clinical data are available for 32 of the 33 US prostatic cancer patients and 17 of the 21 US control patients; complete hormone analyses are available for 29 of the 33 US cancer patients, two of the nine Nigerian cancer patients, and 17 of the 21 US control patients. Tissue sections from either transurethral resection or perineal needle biopsy or both were studied in nine Nigerian and 38 US cancer patients. Epidemiologic questionnaires were completed for 35 US prostatic cancer patients and 21 US controls. Clinical and epidemiologic data from Nigerian patients and controls were not analyzed prior to this report.

The number of patients available for analyses in this preliminary report is small and interpretations based upon this limited data must be made with caution.

The distribution by clinical stage of US patients with carcinoma of the prostate (table 1) shows that 53% (17 patients) are in stages I and II while 47% (15 patients) are in stages III and IV. The interstage age variation is not significant. Plasma testosterone, estrone, and estradiol concentrations in cancer patients were not significantly different from control patients. Table 2 illustrates the concentration of plasma

TABLE 1.—Age distribution and clinical stage of US patients with prostatic carcinoma

		Age (yrs)			
Stage	No. of patients	Mean	Median	Standard deviation	Range
I	10	64.6	65.0	5.3	55–74
II	7	69.3	70.0	7.7	56–74
III	6	73.3	75.5	11.2	60–88
IV	9	71.0	74.0	9.1	60–87
Total	32	69.1	67.5	8.2	55–88
Control	17	65.6	64.0	9.6	53–90

TABLE 2.—Plasma testosterone concentration in US patients with prostatic cancer

		Plasma testosterone concentration (ng/ml)			
Stage	No. of patients	Mean	Median	Standard deviation	Range
I	9	3.31	2.90	1.62	1.3–5.4
II	7	3.31	3.64	1.68	1.0–5.2
III	6	3.91	3.83	2.19	1.21–7.14
IV	7	4.15	3.77	1.40	2.03–5.90
Total	29	3.64	3.90	1.72	1.0–7.14
Control	17	4.49	4.32	1.51	2.61–9.45

testosterone by clinical stage of the disease. The total serum acid phosphatase level was elevated in all patients in stage III and in six of nine patients in stage IV. Cases were included in stage III with elevated serum acid phosphatase levels when bone scans and skeletal surveys were negative and clinical evidence of local tumor extension existed. The total serum phosphatase level was not elevated in any patients in stages I or II.

Tissue was examined from all patients with carcinoma of the prostate. The age distribution of these patients (fig 1) showed that the peak for Nigerians was in the 55–64 age group which is a decade earlier than for the US blacks in Washington, DC. The histologic grade of the tumor (fig 2) tended to be high (more undifferentiated) and the tumor foci more numerous in the Nigerian patients.

Preliminary epidemiologic data from 21 matching pairs of US patients and controls indicate that an association exists between the following variables and prostatic cancer: (*a*) black/black-American Indian admixture, (*b*) later age of puberty, and (*c*) later age of first coitus. Table 3 shows that five of 18 cancer patients and none of the controls indicated that one of their parents or grandparents was American Indian. On the average, prostatic cancer patients reached puberty 1.63 years later than controls and were 3.11 years older than controls when they had their first coitus. These

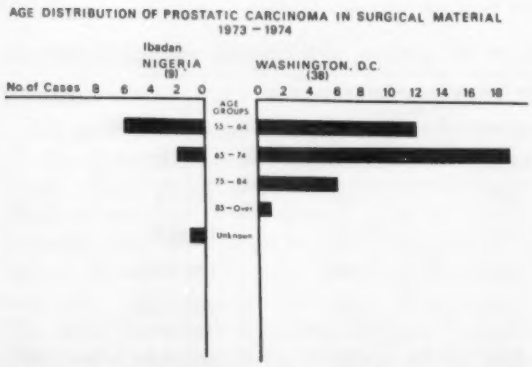

FIGURE 1.—Age distribution of prostatic carcinoma in surgical material (1973–1974).

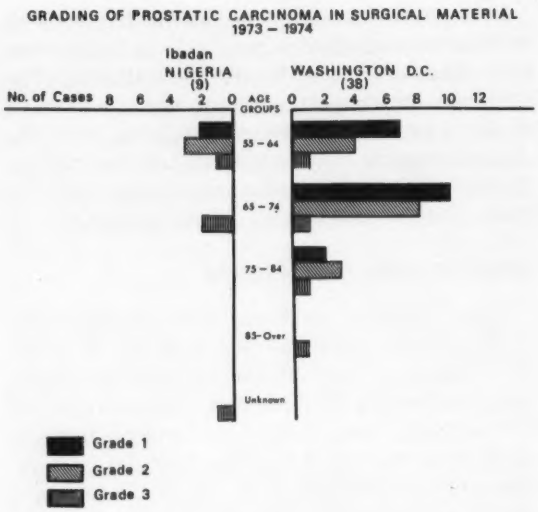

FIGURE 2.—Grading of prostatic carcinoma in surgical material (1973–1974).

TABLE 3.—Racial admixture*

Control	*Patient*		
	Black	Black/American Indian	
Black	13	5	18
Black/American Indian	0	0	0
	13	5	18

*$\chi_1^2 = 5$; $P < 0.05$.

TABLE 4.—Incidence of prostatic carcinoma in necropsy cases

	Ibadan, Nigeria	Washington, DC
Total No. of cases	30	97
Cases without carcinoma	28	78
Cases with carcinoma	2 (67/1000)	19 (196/1000)
Cases with microcarcinoma	1	6
Cases with invasive carcinoma	1	13

variables were statistically significant at $P < 0.05$.

Thirty necropsy cases from Ibadan, Nigeria, and 97 necropsy cases from Washington, DC, were analyzed. The incidence of carcinoma in the US material was 196 of 1000 men as contrasted to 67 of 1000 men in the Nigerian material (table 4). Although the number of cases of carcinoma was small, invasive carcinoma was twice as common as microcarcinoma (incidental) in the US material. The median ages of the necropsy cases from Nigeria and the US were 42.5 and 56.5 years respectively. The median age of cases without carcinoma was 41.7 years in Nigeria and 53.7 years in the US. The median age of cases with carcinoma was 50.0 years in Nigeria and 68.3 years in the US. The median age for microcarcinoma (65.0) was 5 years less than for invasive carcinoma (70.0) in the US material.

APPENDIX 1.—Characterization of prostatic carcinoma among blacks: epidemiology form, USA

Interviewer's No. _____
Hospital identification No. _____
Cancer Prostate Research Grant case No. _____
Patient ___
Control ___
Freedmen's Hospital ___
DC General Hospital ___
Last name _____ Initial ___
Date of birth _____
Age:
1. Estimated by interviewer ___
2. Real ___

Race (up to grandparents):

1. Pure American black ___
2. Pure African black ___
3. Black/Caucasian ___
4. Black/Spanish ___
5. Black/Oriental ___
6. Black/American Indian ___
7. Other (specify) _____
8. Don't know (no answer) ___

Place of longest residence:

State _____
Rural (town with population < 15,000) ___
Urban (city with population > 15,000) ___

Education (maximum):

1. Less than junior high (specify grade) ___
2. Junior high ___
3. High school ___
4. College (undergraduate) ___
5. Graduate degree received ___
6. Don't know (no answer) ___

Occupation (longest):

No. of years ___

I. **Classification**

A. Professional
1. Physician, dentist ___
2. Attorney, judge, etc ___
3. Engineer (mechanical, electrical, civil, etc) ___
4. Top executive, manager, senior civil servant ___
5. University lecturer ___
6. Armed forces and police (above captain) ___
7. Other (specify) _____

B. Paraprofessional
1. Accountant ___
2. Draftsman ___
3. Medical paraprofessional ___
4. Teacher ___
5. Technologist ___
6. Businessman (other than top manager) ___
7. Civil service employee (other than senior) ___
8. Armed forces and police commissioned officer (below captain) ___
9. Other (specify) _____

C. Skilled worker
1. Secretary, clerk ___
2. Machine operator ___
3. Truck or bus driver ___
4. Mechanic ___
5. Electrician ___
6. Plumber ___
7. Armed forces and police (less than officer) ___
8. Printer, painter, photographer ___
9. Merchant marine ___
10. Bricklayer ___
11. Barber/hair dresser ___
12. Butcher ___
13. Salesman ___
14. Other (specify) _____

D. Unskilled worker
1. Porter ___
2. Petty trader ___
3. Janitor ___
4. Messenger ___
5. Construction helper ___
6. Night watchman ___
7. Apprentice to skills above ___
8. Other (specify) _____

E. Farmer ___

II. **Activity involved**

A. Sitting
1. Office, shop, etc ___
2. Vehicle (car, truck, plane, etc) ___

B. Standing (sales clerk, etc) ___

C. Walking (mailman, itinerent street vendor, conductor, waiter, office messenger, etc) ___

D. Straining (porter, trumpet musician, etc) ___

E. No answer ___

III. **Industrial exposure**

Yes _____ (if yes complete entire section)
No _____
Don't know (no answer) _____

A. Physical agents
1. Heat (eg, baker) ___
2. Sound (excess noise) ___
3. Vibration (eg, drilling) ___
4. Pressure (diver, pilot) ___

B. Organic compounds
1. Fuel oil and lubricants (eg, mechanic, service station attendant) ___

2. Paints and paint solvents (painter) ___
3. Dry cleaning fluids (laundry worker) ___
4. Dyes (dye worker) ___
5. Printing chemicals (printer) ___
6. Photography chemicals (developer) ___
7. Pesticides (insecticide sprayer or exterminator) ___
8. Other (specify) _____

C. Hard rock or mineral dusts
1. Silica (rock cutter, miner, road builder, pottery maker, etc) ___
2. Asbestos (pipe fitter, insulator, etc) ___
3. Other (specify) _____

D. Plant dusts
1. Cotton ___
2. Wood ___
3. Other (specify) _____

E. Dusts and fumes of metals
1. Aluminum ___
2. Beryllium ___
3. Cadmium ___
4. Chromium ___
5. Lead ___
6. Manganese ___
7. Mercury ___
8. Other (specify) _____

F. Toxic and irritant gases (specify) _____

G. Ionizing radiation
1. Radiologists and X-ray technicians ___
2. Radioactive isotope handlers ___
3. Mining radioactive ores ___
4. Other (specify) _____

IV. Income

A. Annual
1. < 2000 ___
2. 2000–3999 ___
3. 4000–5999 ___
4. 6000–7999 ___
5. 8000–9999 ___
6. 10,000–11,999 ___
7. 12,000–13,999 ___
8. 14,000–15,999 ___
9. 16,000–17,999 ___
10. 18,000–19,999 ___
11. 20,000–24,999 ___
12. > 25,000 ___

B. Average annual income in the past 20 years:
1. < 2000 ___
2. 2000–3999 ___
3. 4000–5999 ___
4. 6000–7999 ___
5. 8000–9999 ___
6. 10,000–11,999 ___
7. 12,000–13,999 ___
8. 14,000–15,999 ___
9. 16,000–17,999 ___
10. 18,000–19,999 ___
11. 20,000–24,999 ___
12. > 25,000 ___
13. No answer ___

Religion:

A. Christian
1. Protestant ___
a. Anglican ___
b. Methodist ___
c. Baptist ___
d. Unitarian ___
e. Jehovah's Witness ___
2. Catholic ___

B. Muslim ___

C. Other (specify) _____

D. No answer ___

Marital status:

A. Present status
1. Never married ___
2. Married ___
3. Separated ___
4. Divorced ___
5. Widowed ___
6. Common law marriage ___
7. No answer ___
8. No. of yrs (items 2–6) ___

B. Maximum No. of sexual partners ever had in 1 mo ___

C. Total No. of marriages (including common law marriage) ___

D. Fertility
1. Gravity (total No. of pregnancies in spouse[s]) ___
2. Parity (total No. of live-born children) ___
3. Don't know (no answer) ___

Sexual history:

I. Heterosexual

A. Age of puberty
1. < 10 ___
2. 10–12 ___
3. 13–15 ___
4. 16–18 ___
5. ≥ 19 ___
6. Don't know ___

B. Full sexual arousal (ejaculation)
1. < 10 ___
2. 10–12 ___
3. 13–15 ___
4. 16–18 ___
5. ≥ 19 ___
6. Don't know ___

C. Appearance of pubic hair
1. < 10 ___
2. 10–12 ___
3. 13–15 ___
4. 16–18 ___
5. ≥ 19 ___
6. Don't know ___

D. Beard growth (started shaving)
   1. < 10 ___
   2. 10–12 ___
   3. 13–15 ___
   4. 16–18 ___
   5. ≥ 19 ___
   6. Don't know ___

E. Change of voice
   1. < 10 ___
   2. 10–12 ___
   3. 13–15 ___
   4. 16–18 ___
   5. ≥ 19 ___
   6. Don't know ___

F. Age of first coitus
   1. < 10 ___
   2. 10–12 ___
   3. 13–15 ___
   4. 16–18 ___
   5. 19–22 ___
   6. 23–25 ___
   7. 26–29 ___
   8. ≥ 30 ___
   9. Don't know ___

G. Age sexual activity stopped
   1. Still active ___
   2. < 30 ___
   3. 30–39 ___
   4. 40–49 ___
   5. 50–54 ___
   6. 55–59 ___
   7. 60–64 ___
   8. 65–69 ___
   9. ≥ 70 ___
   10. Don't know ___

H. Sexual activity during yrs prior to present illness

	Yrs					
	None	0–5	5–10	10–19	20	Don't know
1. Frequency of coitus/mo	___	___	___	___	___	___
2. No. of different sexual partners/mo	___	___	___	___	___	___
3. Avg duration of single intercourse (in min)	___	___	___	___	___	___
4. No. of ejaculations in a single intercourse	___	___	___	___	___	___
5. Masturbation (No./mo)	___	___	___	___	___	___
6. Oral sex (No./mo)	___	___	___	___	___	___
7. Anal sex (No./mo)	___	___	___	___	___	___
8. Practice of coitus interruptus (No./mo)	___	___	___	___	___	___
9. Interest in more coitus than occurred (yes or no)	___	___	___	___	___	___
10. Habitual use of aphrodesiacs (yes or no)	___	___	___	___	___	___

I. Source and type of aphrodesiac, if user
   A. Drug store
      1. Vitamins ___
      2. Hormones ___
   B. Peddler
      1. Liquid ___
      2. Powder ___
   C. Self prepared (specify) _____
   D. Other (specify) _____

J. Impotence (unable to carry intercourse at all for 1 mo or longer)
   1. Yes ___
   2. No ___
   3. No. of times ___
   4. Duration of longest period of impotence
      a. 1–5 mos ___
      b. 6 mos ___
      c. 1–2 yrs ___
      d. > 2 yrs ___

K. Coitus while partner having menses
   1. No ___
   2. Yes ___
   3. Maximum No. of times/yr ___

II. Homosexual
   A. No ___
   B. Yes ___
   C. Active ___
   D. Passive ___

Interviewer's rating of patient's response to questions on sexual habits
   1. Reliable ___
   2. Exaggerating ___
   3. Minimizing ___
   4. Withholding facts ___

Hair distribution:

A. Baldness
   1. None ___
   2. Partial ___
   3. Complete ___

B. How often necessary to shave to keep clean shaven
   1. Daily ___
   2. Every other day ___

3. Every third day ___
4. Less often ___

C. Hair in pelvic region
1. None ___
2. Scant (little hair on mons pubis) ___
3. Normal (pubic and genital region) ___
4. Very hairy (all over abdomen, pubis, and thighs) ___

D. Hair in chest region
1. None ___
2. Scant (few around nipple or in center of chest) ___
3. Normal (between nipple and furtherout) ___
4. Very hairy (bushy) ___

Circumcision:

1. No ___
2. Yes ___
3. What age ___

Genitourinary system history:

A. Dysuria
1. Smarting sensations
a. Yes ___
b. No ___
2. Suprapubic pain
a. Yes ___
b. No ___

B. Nocturia (does patient wake up at night to void?)
1. No ___
2. Yes ___
3. How many times/night ___

C. Hematuria
1. Initial (at start of stream)
a. None ___
b. Yr of onset ___
c. Duration ___
2. Total (throughout stream)
a. None ___
b. Yr of onset ___
c. Duration ___
3. Terminal (at end of stream)
a. None ___
b. Yr of onset ___
c. Duration ___

D. Incomplete voiding sensation
1. No ___
2. Yes ___
a. Urgency ___
b. Hesitancy ___
c. Chronic retention ___
d. Acute retention ___
e. Interruption of stream ___
f. Loss of force and decrease in caliber ___
g. Back pain ___
h. Other (specify) _____

E. Diseases
1. Gonorrhea
a. None ___
b. Yr of onset ___
c. Duration ___
d. Treated after antibiotics came into use ___
e. Treated before antibiotics came into use ___
2. Syphilis
a. None ___
b. Yr of onset ___
c. Duration ___
d. Treated after antibiotics came into use ___
e. Treated before antibiotics came into use ___
3. Urethral stricture
a. None ___
b. Yr of onset ___
c. Duration ___
d. Dilation ___
e. No dilation ___
4. Benign prostatic hyperplasia
a. None ___
b. Yr of onset ___
c. Duration ___
d. Operation
i. Yes ___
ii. No ___
iii. Don't know ___
5. Urinary calculi
a. Kidney
1. None ___
2. Yr of onset ___
3. Duration ___
4. Don't know ___
b. Bladder
1. None ___
2. Yr of onset ___
3. Duration ___
4. Don't know ___
c. Prostate
1. None ___
2. Yr of onset ___
3. Duration ___
4. Don't know ___

History of other illnesses:

I. Heart
A. Arteriosclerotic
1. None ___
2. Yr of onset ___
3. Duration ___
4. Don't know ___

B. Rheumatic
1. None ___
2. Yr of onset ___
3. Duration ___
4. Don't know ___

C. Congenital
1. None ___

2. Yr of onset ___
3. Duration ___
4. Don't know ___

D. Infectious
1. None ___
2. Yr of onset ___
3. Duration ___
4. Don't know ___

E. Hypertension
1. None ___
2. Yr of onset ___
3. Duration ___
4. Don't know ___

II. Respiratory

A. Chronic obstructive
1. Emphysema
a. None ___
b. Yr of onset ___
c. Duration ___
d. Don't know ___
2. Asthma
a. None ___
b. Yr of onset ___
c. Duration ___
d. Don't know ___

B. Infectious
1. Tuberculosis
a. None ___
b. Yr of onset ___
c. Duration ___
d. Don't know ___
2. Chronic bronchitis
a. None ___
b. Yr of onset ___
c. Duration ___
d. Don't know ___

III. Diabetes mellitus

A. None ___
B. Yr of onset ___
C. Duration ___
D. Don't know ___

IV. Cirrhosis of the liver

A. None ___
B. Yr of onset ___
C. Duration ___
D. Don't know ___

V. Hepatitis (jaundice)

A. None ___
B. Yr of onset ___
C. Duration ___
D. Don't know ___

VI. Other major illnesses (specify)

No ___
Yes ___

A. _____
1. Yr of onset ___
2. Duration ___

B. _____
1. Yr of onset ___
2. Duration ___

Past radiologic, surgical, or hormonal treatment for diseases other than present illness:

A. Surgery
1. None ___
2. Yr of exposure ___

B. Radiotherapy
1. Diagnostic
a. None ___
b. Yr of exposure ___
c. Duration of exposure ___
d. Don't know ___
2. Therapeutic
a. None ___
b. Yr of exposure ___
c. Duration of exposure ___
d. Don't know ___

C. Hormonal
1. Pituitary
a. None ___
b. Yr of exposure ___
c. Duration of exposure ___
d. Don't know ___
2. Pancreas
a. None ___
b. Yr of exposure ___
c. Duration of exposure ___
d. Don't know ___
3. Thyroid
a. None ___
b. Yr of exposure ___
c. Duration of exposure ___
d. Don't know ___
4. Suprarenal
a. None ___
b. Yr of exposure ___
c. Duration of exposure ___
d. Don't know ___
5. Testis
a. None ___
b. Yr of exposure ___
c. Duration of exposure ___
d. Don't know ___
6. Ovary
a. None ___
b. Yr of exposure ___
c. Duration of exposure ___
d. Don't know ___

Family history (first-line blood relatives):

A. Cancer
1. Urogenital ___

2. Respiratory ____
3. Gastrointestinal ____
4. Hemopoietic ____
5. CNS ____
6. Don't know ____

B. Endocrine disease
1. Goiter
a. Father ____
b. Mother ____
c. Siblings ____
d. Don't know ____
2. Diabetes
a. Father ____
b. Mother ____
c. Siblings ____
d. Don't know ____

C. Genitourinary diseases
1. Stones
a. Father ____
b. Mother ____
c. Siblings ____
d. Don't know ____
2. Prostatic disease
a. Father ____
b. Siblings ____
c. Don't know ____
3. Kidney disease
a. Father ____
b. Mother ____
c. Siblings ____
d. Don't know ____

D. Other disease(s) (specify)
1. _____
a. Father ____
b. Mother ____
c. Siblings ____

Habits:

I. Drinking (key: light = occasionally, < 2 drinks/day; moderate = daily, not > 3 drinks/day)

A. Beer (1 drink = 1 pint)
1. Never ____
2. Light ____
3. Moderate ____
4. Heavy ____
5. No answer ____

B. Wine (1 drink = 1 pint)
1. Never ____
2. Light ____
3. Moderate ____
4. Heavy ____
5. No answer ____

C. Hard liquor (1 drink = 1 oz)
1. Never ____
2. Light ____
3. Moderate ____
4. Heavy ____
5. No answer ____

D. Home-made alcoholic beverage
1. Distilled
a. Never ____
b. Light ____
c. Moderate ____
d. Heavy ____
e. No answer ____
2. Other (fermented)
a. Never ____
b. Light ____
c. Moderate ____
d. Heavy ____
e. No answer ____

II. Chewing

A. Tobacco
1. No ____
2. Amount/day (oz) ____
3. Duration ____

B. Colanut
1. No ____
2. Amount/day (oz) ____
3. Duration ____

C. Bitter nut
1. No ____
2. Amount/day (oz) ____
3. Duration ____

D. Other (specify) _____
1. Amount/day (oz) ____
2. Duration ____

III. Smoking

A. Cigarettes
1. No ____
2. < 5/day ____
3. 5–9/day ____
4. 10–19/day ____
5. 1 pack/day ____
6. > 1 but < 2 packs/day ____
7. ≥ 2 packs/day ____

B. Cigars
1. No ____
2. 1–2/day ____
3. 3–4/day ____
4. ≥ 5/day ____

C. Pipe
1. No ____
2. Yes ____
3. No. of oz/day ____

History relative to sexual partner(s):

A. Contraceptives (most frequently used method)
1. None ____
2. Condom ____
3. Jelly/foam/creams ____
4. Diaphragm ____
5. IUD ____

6. Pill ____
7. Douche ____
8. Don't know ____

B. Genital illness
1. Gonorrhea
a. None ____
b. Yr of onset ____
c. Duration ____
d. No answer ____
2. Cervicitis and/or vaginitis (heavy discharge)
a. None ____
b. Yr of onset ____
c. Duration ____
d. No answer ____
3. Syphilis
a. None ____
b. Yr of onset ____
c. Duration ____
d. No answer ____

C. Cancer
1. Cervix
a. None ____
b. Yr of onset ____
c. Duration ____
d. No answer ____
2. Uterus
a. None ____
b. Yr of onset ____
c. Duration ____
d. No answer ____

Food:

I. What item(s) on the following list do you *not* like to eat?

A. Bread (wheat) ____
B. Rice ____
C. Yams
1. Whole boiled ____
2. Fried ____
D. Beans (whole boiled) ____
F. Potato ____
F. Fruit ____
G. Breakfast cereal ____
H. Milk ____
I. Corn ____
J. Eggs ____
K. Meat
1. Beef ____
2. Lamb ____
3. Goat ____
L. Chicken ____
M. Seafood
1. Fish ____
2. Oysters ____
3. Clams ____
4. Other ____

II. How do you like your favorite foods prepared?

A. Fried
1. Yes ____
2. No ____
3. No answer ____

B. Boiled
1. Yes ____
2. No ____
3. No answer ____

C. Baked
1. Yes ____
2. No ____
3. No answer ____

D. Smoked
1. Yes ____
2. No ____
3. No answer ____

E. Pickled
1. Yes ____
2. No ____
3. No answer ____

F. Fermented
1. Yes ____
2. No ____
3. No answer ____

III. What is the material of the containers of homemade alcohol (moonshine or wine)?

A. Iron ____
B. Tin ____
C. Glass (bottle) ____
D. Plastic ____
E. Enamel-lined ____
F. Aluminum ____
G. Other (specify) _____
H. Don't know ____

III. What is the material from which most of your eating utensils are made?

A. Ceramic ____
B. Enamel-lined ____
C. Silver ____
D. Plastic ____
E. Paper ____
F. Stainless steel ____
G. Other (specify) _____
H. Don't know ____

IV. How many cans of food and drink consumed/wk?

A. Vegetables ____
B. Fruits ____
C. Meats ____
D. Fish ____
E. Beer ____
F. Soft drinks ____

V. What spices do you most commonly add to your food?

A. Black pepper ____
B. Garlic ____
C. Tumeric ____
D. Salt ____
E. Red pepper ____
F. Other (specify) _____

VI. What food additives do you usually use for—

A. Color
1. None ____
2. Specify ____
3. Don't know ____

B. Flavor
1. None ____
2. Specify ____
3. Don't know ____

C. Tenderness
1. None ____
2. Specify ____
3. Don't know ____

VII. What fat do you use most often?

A. Lard ____
B. Olive oil ____
C. Oleomargarine ____
D. Peanut oil ____
E. Butter ____
F. Crisco oil (or similar fat) ____
G. Other (specify) _____

VIII. What raw foods do you commonly eat? (check one or all)

A. Vegetables ____
B. Fruits ____
C. Roots (specify)_____
D. Eggs ____
E. Oysters ____
F. Other (specify) _____

IX. Which of the following water extracts do you frequently drink?

A. Tea ____
B. Cocoa (chocolate) ____
C. Coffee ____
D. Coca cola, Pepsicola ____

X. Do you usually eat any of the following foods half-cooked (rare)?

A. Beef ____
B. Lamb ____
C. Goat ____
D. Pork ____
E. None ____
F. No answer ____

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Virologic and Immunologic Studies of Human Prostatic Carcinoma[1]

Leon Dmochowski, Koshi Maruyama, Yuji Ohtsuki, Gabriel Seman, James M. Bowen, William A. Newton, and Douglas E. Johnson[2,3]

SUMMARY

Morphologic, tissue culture, immunologic, and biochemical methods have been used in an attempt to detect and characterize oncogenic viruses or their subviral components in cells derived from human prostatic carcinoma (PrCa) or benign prostatic hyperplasia (BPH). Electron microscopy was used to characterize the ultrastructural features of normal and neoplastic prostatic tissue. Examination of specimens of prostatic tissue from 34 patients with PrCa, ten patients with BPH, and three patients with bladder tumor (BT) revealed the presence of particles resembling type-C virus in three cases of PrCa and structures resembling budding type-C virus particles in one case of BPH. Fifty human prostatic tissue specimens have been set in tissue culture, of which 30 have been successfully grown for varying periods of time. Of 20 currently active cultures, nine consist primarily of epithelial cells. Immunofluorescence and mixed hemadsorption tests of cells derived from benign and malignant prostatic tissue and sera derived from patients with PrCa, BPH, BT, and other types of tumors, and from normal donors revealed that sera from patients with PrCa, BPH, or BT contain antibodies to antigens in cells derived from PrCa, BPH, or BT. The nature of these antigen-antibody reactions is under study. Initial biochemical studies have not detected reverse transcriptase in the tissue culture fluid from a small number of sparsely growing PrCa cultures nor specific gene sequences homologous to murine leukemia virus-Rauscher genomic RNA in preparations of either normal or malignant prostatic cell DNA. The results of these preliminary studies have demonstrated the applicability of the techniques employed to the study of the relationship of viruses to human PrCa and have provided a number of promising leads for further investigation.

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Morphologic, biologic, immunologic, and biochemical studies of animal model systems have established the etiologic role of viruses in neoplasms of various species of animals (1). These viruses represent members of both RNA and DNA virus groups (1). Application of similar experimental approaches has provided

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[2] Department of Virology (Drs. Dmochowski, Maruyama, Ohtsuki, Seman, Bowen, and Newton) and Department of Surgery (Dr. Johnson), The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute at Houston.

[3] *Reprint requests to*: Leon Dmochowski, MD, Department of Virology, The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute, 6723 Bertner Dr, Houston, Tex 77025.

considerable indirect evidence implicating viruses in the origin of at least some human tumors, such as leukemia, lymphoma, sarcoma, nasopharyngeal carcinoma, and breast carcinoma (2). However, there is no information at present pertaining to the possible involvement of viruses in benign and malignant changes in the prostate gland of man.

The present studies were undertaken in an attempt to detect either RNA or DNA tumor viruses or their subviral components in cells derived from prostatic carcinoma (PrCa) or benign prostatic hyperplasia (BPH). In these studies a multifaceted approach was undertaken, utilizing morphologic, tissue culture, immunologic, and biochemical methods. It should be noted that the results to be described are preliminary but may serve to indicate some of the lines of research on possible relationships of viruses to human PrCa.

MORPHOLOGIC STUDIES

A number of ultrastructural studies of both benign and malignant prostatic tissues have been reported but no virus particles have been demonstrated, except for a case in which herpes-type virus particles have been observed by Centifanto and his associates (3).

In the present study, we examined surgical or biopsy prostatic tissue specimens from 34 patients with PrCa, ten patients with BPH, and three patients with bladder tumor (BT). Four specimens from the seminal vesicles of one patient with adenocarcinoma and three nontumorous cases have been examined. Further, tissue culture specimens derived from five patients with PrCa and two patients with BPH, and three specimens of the MA–160 culture (derived from BPH) (4) have been studied. Specimens of prostatic tissues and their tissue cultures were prepared for electron microscopy by methods previously described (5). The following observations have been made.

Carcinoma of the Prostate

Five PrCa specimens biopsied by transurethral resection consisted of autolyzed tissues and were not suitable for study. No epithelial elements could be detected by paragon staining in each of ten to 15 blocks from five cases of PrCa. In 22 specimens of PrCa, cancer cells showed irregular nuclei with large nucleoli, poorly developed cellular organelles, and weak secretory activity as evidence of undifferentiation (fig 1*a*).

In two cases of well-differentiated adenocarcinoma as well as in BPH marked interdigitation of the cell membrane was observed (fig 1*b*). In adenocarcinoma, it was occasionally impossible to detect a clear basal cell layer of the glands. In the nests of undifferentiated carcinoma cells, the surrounding basal layer was partially missing.

Particles 90–130 nm in diameter, resembling type–C oncornavirus particles, were found in intracisternal spaces (figs 1*c*, 1*d*, and 2*a*) and apparently in the cytoplasm of cells from two cases of undifferentiated carcinoma and in one case of well-differentiated PrCa (figs 2*b*–2*d*). It may be of interest to mention that in five cases of undifferentiated PrCa, bundles of tubules were observed in the cytoplasm of the cancer cells (fig 2*e*). These bundles (straight, wavy, or curved) were usually located in the perinuclear region and were 50–70-nm wide and 1μ–10μ long tubules, running parallel. In cross-section of these bundles, with no limiting membrane and no connections with other organelles, each tubule 50–70 nm in diameter appeared in a crystal-like arrangement. For the purpose of clarifying the nature of these structures, RNase digestion will be carried out.

Mitotic figures of cancer cells were observed in only three cases of undifferentiated carcinoma. In the nucleus of cancer cells, fibrillar bundles have been observed in two cases of undifferentiated carcinoma (fig 3*a*). Unusual, multilayered, large nuclear inclusions were seen in two cases of undifferentiated carcinoma (figs 3*b* and 3*c*). Degenerating mitochondria in four cases of undifferentiated carcinoma were also observed. Single cilia with or without rootlet fibers with 30–50-nm wide periodic striation have been seen in the cytoplasm of cells from 13 undifferentiated cases and one well-differentiated case of carcinoma (fig 3*d*).

Intracellular desmosomes have been found in the cells of three cases of undifferentiated carcinoma (fig 4*a*). Unusual mitochondria with lamellar, circular, or crystal-like cristae have

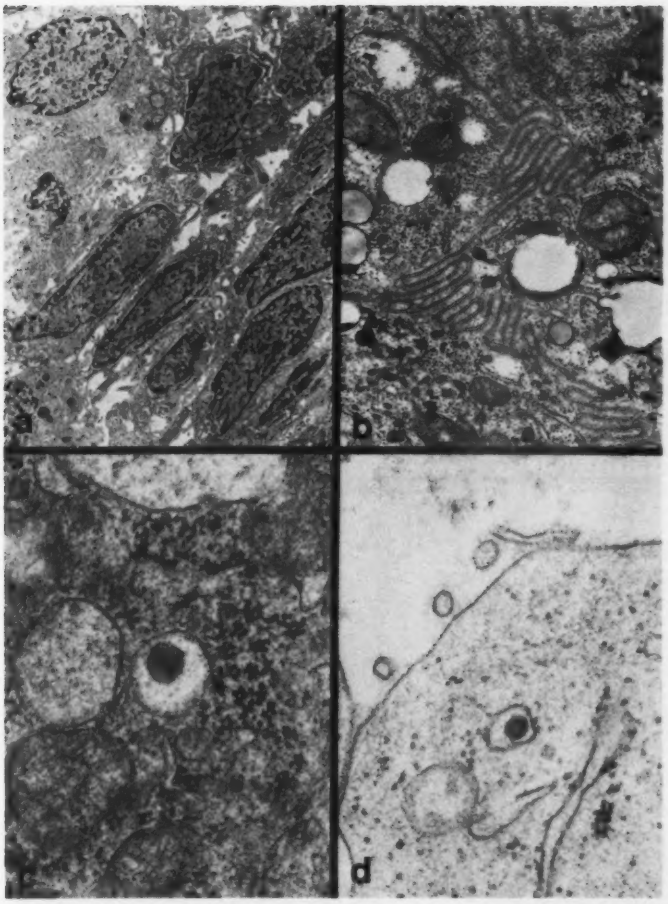

FIGURE 1.—(*a*) Undifferentiated PrCa. Appearance of cancer cell cluster at low magnification (× 6000). (*b*) Undifferentiated PrCa. Marked interdigitation of cell membranes may be seen (× 20,000). (*c*) Undifferentiated PrCa. Double-layered intracisternal particle, approximately 130 nm in diameter (× 55,000). (*d*) Undifferentiated PrCa. Intracisternal particle with electron-lucent center, approximately 90 nm in diameter (× 55,000).

been observed in five cases of undifferentiated carcinoma (figs 4*b* and 4*c*). Unknown structures with periodic striation were also observed in one case of undifferentiated carcinoma (fig 4*d*), annulate lamellae in one case, intracellular duct-like structures, and many lipid-droplets of various sizes in three cases.

Benign Prostatic Hyperplasia

The appearance of BPH tissue at lower magnification may be seen in figures 5*a* and 5*b*. Peculiar intracytoplasmic bundles of tubules, found in five cases of PrCa, were observed in one of five cases of BPH. Single cilia with or without rootlet fibers have been found in all five cases. Intracellular canals, vesicular aggregation of Golgi apparatus, and electron-dense, interlacing, cord-like structures in the large glandular spaces have also been observed. Structures resembling budding of type-C virus particles were also seen in one case (figs 5*c* and 5*d*).

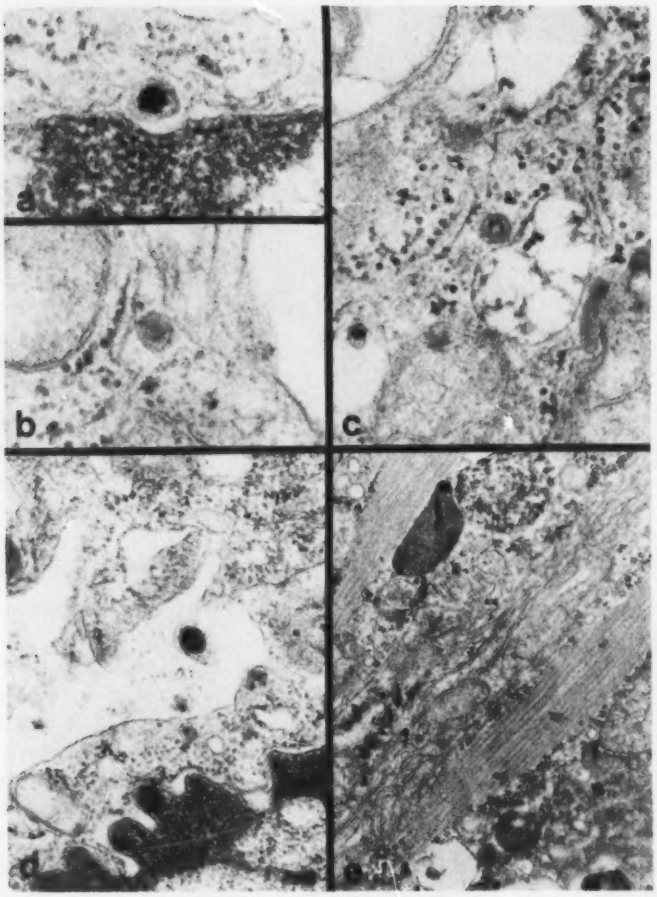

FIGURE 2.—(*a*) Undifferentiated PrCa. Particle with electron-dense core, approximately 130 nm in diameter, in perinuclear space of a cancer cell (× 75,000). (*b*) Undifferentiated PrCa. Intracytoplasmic particle with a dense central core, approximately 100 nm in diameter, present in a cancer cell (× 75,000). (*c*) Well-differentiated PrCa. Intracytoplasmic double-layered particle with electron-lucent center, approximately 110 nm in diameter, present in a cancer cell (× 60,000). (*d*) Undifferentiated PrCa. Multilayered granule, approximately 150 nm in diameter, present in a cellular process (× 40,000). (*e*) Undifferentiated PrCa. Longitudinal section of intracytoplasmic bundles of 50–70-nm wide tubules in a cell (× 20,000).

Bladder Tumor

In each of the three cases of BT from which prostatic tissue was examined, peculiar bundles of tubules, intracellular desmosomes, and single cilia (fig 5*e*) were detected.

Seminal Vesicles

In one tumorous and one nontumorous case, single cilia with rootlet fibers but not virus particles were observed.

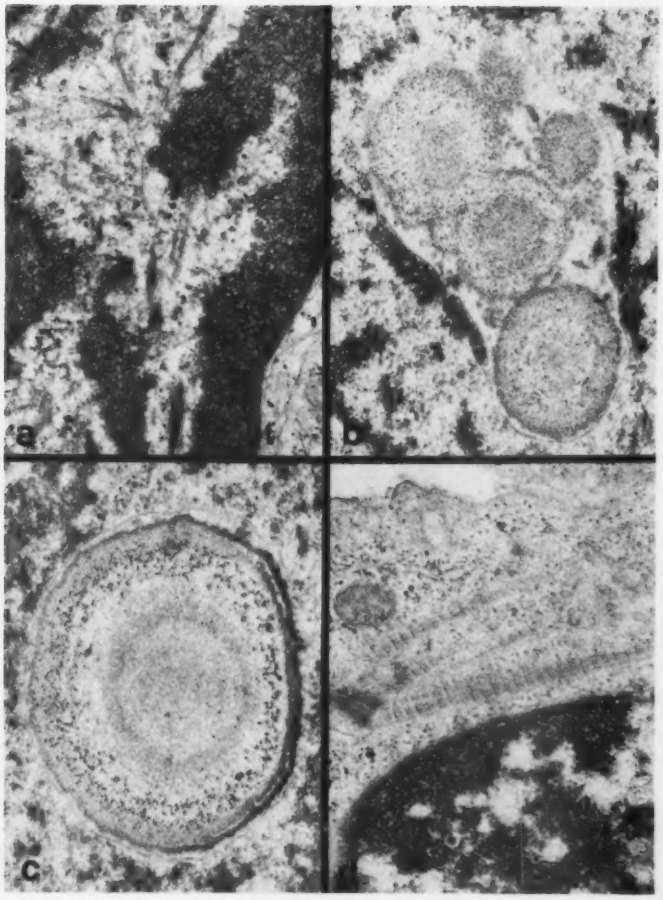

FIGURE 3.—(*a*) Undifferentiated PrCa. Irregular intranuclear fibrillar bundles in a cell nucleus (× 30,000). (*b*) Undifferentiated PrCa. Several inclusions may be seen in a cell nucleus (× 30,000). (*c*) Undifferentiated PrCa. Multilayered inclusion in a cell nucleus (× 30,000). (*d*) Undifferentiated PrCa. Basal body with rootlet fibers showing 30–50-nm periodic striation in a cancer cell (× 30,000).

Tissue Culture Specimens Derived From Prostatic Tumors

Lower magnification of prostatic adenoma cells or the MA-160 cell line may be seen in figures 6*a* and 6*b*. In these cells, distinct desmosomes (fig 6*c*), bundles of 30-nm wide microtubules, paired cisternae, annulate lamellae, intracytoplasmic fibrillar bundles, single cilia, and glycogen aggregation could be occasionally observed; no virus particles or pleuropneumonia-like organisms (PPLO) were seen. The other seven tissue culture specimens appeared to be of fibroblastic cell origin. Intranuclear fibrillar bundles, annulated lamellae, large nuclear bodies, and single cilia (fig 6*d*) were observed; no virus particles or PPLO were seen.

Morphologic studies carried out so far have

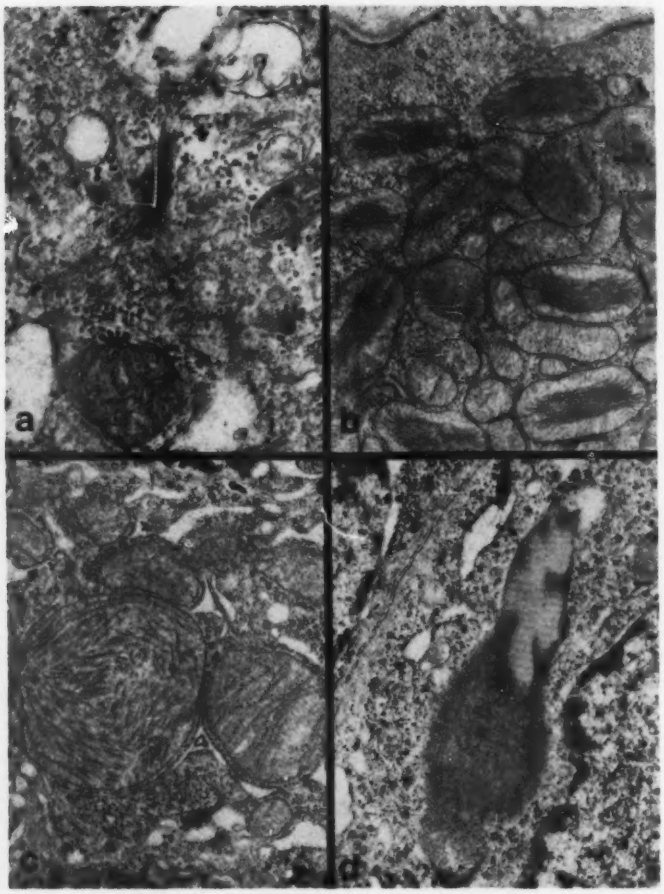

FIGURE 4.—(*a*) Undifferentiated PrCa. Intracellular desmosome present in a cancer cell (× 40,000). (*b*) Undifferentiated PrCa. Lamellar arrangement of cristae in mitochondria of a cell (× 20,000). (*c*) Undifferentiated PrCa. Irregular bundles of small filaments in large mitochondria of a cell (× 20,000). (*d*) Undifferentiated PrCa. Intracytoplasmic partially striated electron-dense structure in a cell (× 25,000).

demonstrated a number of interesting ultrastructural features in specimens of malignant and benign prostatic hypertrophy. Particles resembling type-C virus particles have been observed in three cases of PrCa and budding of virus particles in one case of BPH. No herpesvirus particles have been observed so far.

TISSUE CULTURE STUDIES

Benign and malignant prostatic tissues have been grown in both organs (6) and monolayer culture systems (7,8), but no cell lines of prostatic cancer tissue have as yet been reported. However, a cell line derived from a benign prostatic adenoma (the so-called MA-160) has been described (4).

Thus far, 50 human prostatic tissue specimens (surgical and needle biopsy) have been set in tissue culture, of which 30 have been grown by conventional methods for stationary

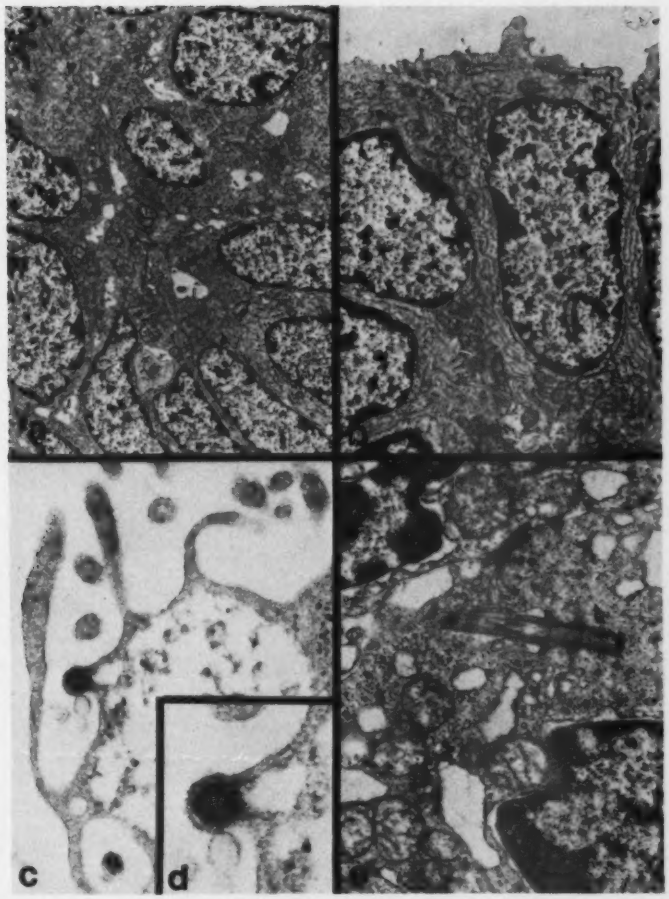

FIGURE 5.—(*a*) BPH. Cells at lower magnification of tangentially sectioned gland (× 4000). (*b*) BPH. A part of papillary projection of hyperplastic gland (× 6000). (*c*) BPH. Particle resembling type-C virus particle, approximately 110 nm in diameter, budding into extracellular space from an epithelial cell (× 40,000). (*d*) Same as fig 5*c*, at higher magnification (× 80,000). (*e*) Infiltrating BT of the prostate. Longitudinal section of single cilium in a cell (× 20,000).

tissue culture. Of the 20 cultures currently active, nine contain areas which consist primarily of epithelial cells. Some of these cultures consisting of epithelial cells have now been maintained for over 3 months. However, they have not been successfully subcultured as yet. Some actively growing fibroblastic cell cultures derived from human PrCa have been used in immunologic studies. In addition, the cell line derived from human prostatic adenoma (MA-160) and reported transformed in vitro (obtained through the courtesy of Dr. M. M. Vincent of Microbiological Associates, Inc.) was used in immunologic studies.

IMMUNOLOGIC STUDIES

A variety of immunologic methods have been employed to demonstrate antigens of different

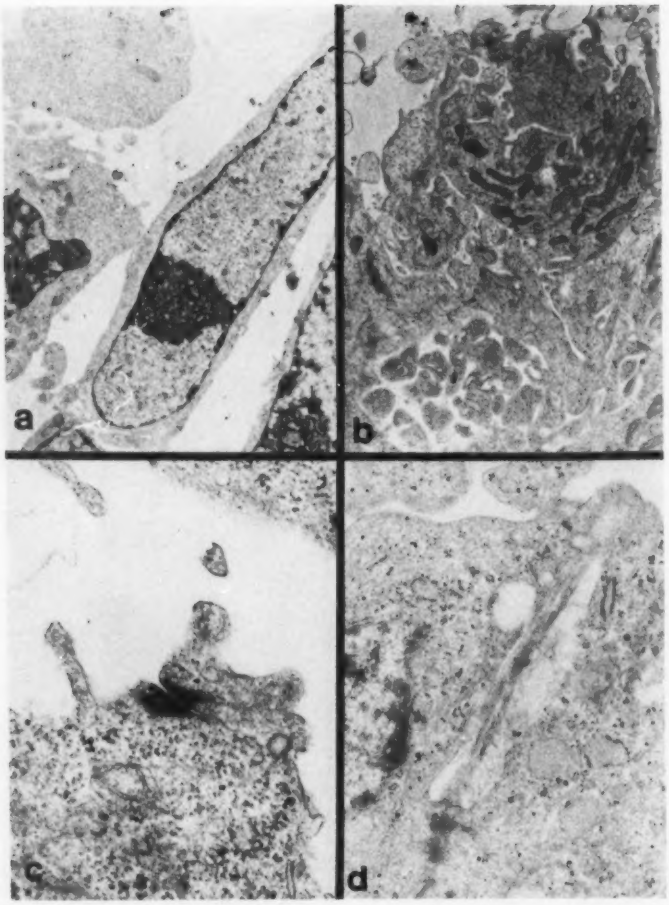

FIGURE 6.—(*a*) Appearance of cells in tissue culture line (MA-160) derived from a prostate adenoma. Culture cells at low magnification (× 6000). (*b*) Cells of tissue culture line (MA-160) at low magnification. Note small desmosomes (× 6000). (*c*) Clone of tissue culture line (MA-160). Distinct desmosome may be seen (× 40,000). (*d*) Tissue culture of cells derived from PrCa. Longitudinally sectioned single cilium projecting into extracellular space (× 25,000).

types in animal and human neoplasia (9).

The indirect fixed immunofluorescence test (10) has been used in the present study to determine whether patients with PrCa develop antibodies to antigens of cells of autologous and/or homologous PrCa.

Sera from 55 patients with PrCa, from four patients with BPH, and from three patients with BT were tested at 1:2 and 1:10 dilutions after heating at 56°C for 30 minutes. Cells from five fibroblastic cell cultures (P-1, P-2, P-6, P-7, and P-9) derived from PrCa tissues and from one epithelial cell culture (MA-160) were tested after fixation in acetone at −76°C. Goat anti-human IgG globulin conjugated with fluorescene isothiocyanate (FITC) was used. Summary results are shown in table 1. At a 1:10 dilution of serum, 29 of 47 sera (62%)

TABLE 1.—Results of fixed immunofluorescence tests*

Cells	No. of sera giving fluorescent reaction at 1:10 dilution/No. tested				
	PrCa	BPH	BT	Other neoplasms*	Normal
P-1 (PrCa)	29/47	2/2	3/3	NT	NT
P-2 (PrCa)	0/50	0/4	0/3	NT	NT
P-6 (PrCa)	8/47	0/2	2/3	NT	NT
P-7 (PrCa)	2/48	0/4	2/3	NT	NT
P-9 (PrCa)	4/55	0/4	0/3	NT	NT
MA-160 (BPH)	37/50	1/4	3/3	9/106	0/55
HeLa	29/31	3/4	3/3	NT	1/39

*NT = not tested.

from carcinoma patients gave cytoplasmic fluorescent reactions with P-1 cells. None of the sera from patients with PrCa reacted with P-2 cells, 17% reacted with P-6 cells, 4% reacted with P-7 cells, and 7% reacted with P-9 cells. MA-160 cells reacted with 74% of sera from patients with PrCa. A greater number of sera from patients with BPH or BT will be tested in the future. It should be pointed out that P-6 and P-9 cells reacted with autologous sera at a 1:10 dilution and cells from P-1, P-2, and P-7 cultures also gave fluorescent reaction with autologous sera at a 1:2 or a 1:10 dilution. Examples of cytoplasmic fluorescence may be seen in figures 7*A* and 7*B*.

Sera from a total of 106 patients with neoplasms other than PrCa, BPH, or BT were tested against cultured transformed human cells derived from prostatic adenoma (MA-160) (*see* table 1). None of 55 normal donor sera reacted with MA-160 cells at a 1:10 dilution. It is of interest that serum from a patient with carcinoma of the uterus reacted with MA-160 cells at a 1:10 dilution. Rabbit anti-herpes simplex virus type 2 serum (HSV-2) gave a titer of 1:128 against MA-160 cells in a preliminary titration by the fixed immunofluorescence test. Testing of a greater number of sera from patients with carcinoma of the uterine cervix is required to investigate possible involvement of HSV-2 in some of the positive fixed immunofluorescence reactions of MA-160 cells with sera from patients with different neoplasms.

Titration of five selected human sera positive with MA-160 cells was carried out by the fixed immunofluorescence test. These sera included serum from a patient with BT and sera from four patients with PrCa. The titer of the serum from the patient with BT was 1:128. The titers of four sera from patients with PrCa varied from 1:10 to 1:128. After absorption with sheep red blood cells and sequentially with guinea pig kidney powder, the titer of the serum from the patient with BT was reduced from 1:128 to 1:10. Similar absorption of sera from patients with PrCa reduced the titer of one serum from 1:128 to 1:2 and the titers of two sera from 1:16 or 1:32 to 1:4. This absorption removed the reactivity of one serum which had a titer of 1:10 before absorption. MA-160 cell antigens that reacted with these human sera are therefore similar, at least in part, to Forssman antigen.

Imprint preparations of prostatic tissue from one patient with urinary BT have been tested by the indirect immunofluorescence test after fixation in cold acetone with sera from seven patients with PrCa and three patients with urinary BT. Three of seven sera from patients with PrCa were positive at a 1:2 serum dilution and were negative at a 1:10 dilution.

Absorption of these sera with human PrCa tissue, benign adenoma tissue, or normal prostate tissue gave inconclusive results due to insufficient amounts of tissues available. In general, the titer of sera from patients with PrCa was most reduced by absorption with

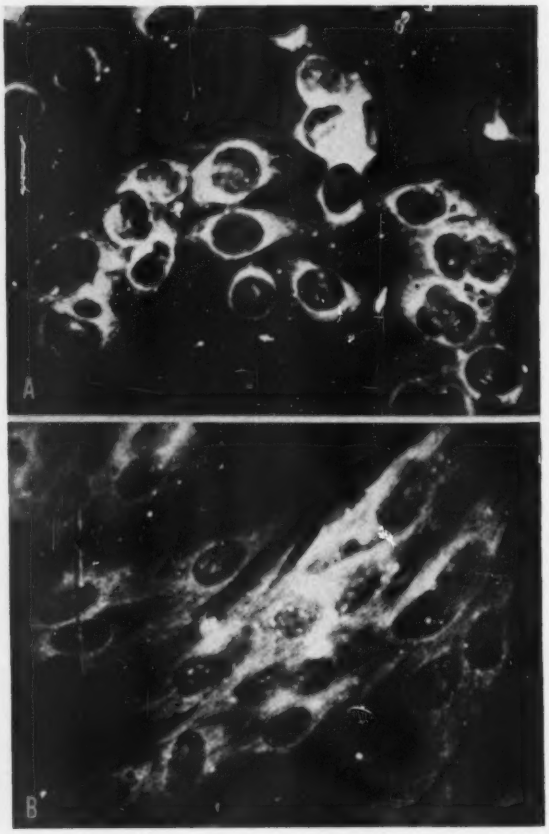

FIGURE 7.—(*A*) Cytoplasmic and perinuclear fluorescence of MA-160 tissue culture cells, treated with serum from a patient with PrCa (dilution 1:2) (× 900). (*B*) Cytoplasmic and perinuclear fluorescence of tissue culture cells derived from prostatic carcinoma (passage 5), treated with autologous serum from a patient with PrCa (dilution 1:2) (× 900).

PrCa tissue. These absorption experiments will be repeated when large amounts of PrCa tissue become available.

Results of recent studies of chromosomes and isoenzyme patterns of MA-160 cells have indicated a possible contamination of MA-160 with HeLa cells (11). In this connection, sera from 38 patients with PrCa or BT were tested by indirect fixed immunofluorescence against HeLa cells. Results are shown in table 1. Thirty-five of 38 sera (92.1%) were positive at a 1:10 dilution. In contrast, only one of 39 sera (2.5%) of normal donors tested gave positive reaction at a 1:10 dilution with HeLa cells.

Immunofluorescence reactivity of MA-160 cells with four selected positive sera from patients with PrCa and with serum from one patient with BT was blocked by goat anti-feline leukemia virus serum. These findings suggest a similarity between antibodies in sera of patients with PrCa and the anti-feline leukemia virus goat serum.

Sera of 89 patients with PrCa, ten patients with BPH, and three patients with BT were tested by mixed hemadsorption (12) against MA-160 cells. Only one serum from a patient with PrCa gave an insignificant titer of 1:4, while all other sera were negative.

The results of immunologic studies carried out so far indicate the presence of antibodies in sera from patients with PrCa against cells derived from their own tumor and tumors from other patients. The nature of these antibodies and the antigens require further careful study.

BIOCHEMICAL STUDIES

The application of the so-called biochemical probes to the study of the relationship of viruses to human neoplasia has indicated that a variety of human tumor cells contain viral genetic information (13). Similar biochemical studies have to our knowledge not been previously applied to the possible viral etiology of human PrCa.

Biochemical studies have been undertaken in search of oncornavirus information in cells of human PrCa. Initial attempts have been directed toward detection of the expression of the viral enzyme (RNA-directed DNA polymerase or reverse transcriptase) in urogenital tumors, and detection of viral-specific DNA sequences integrated into the cellular DNA of prostatic tissue.

Murine leukemia virus, Rauscher strain (MuLV-R), was chosen in the absence of a proven human RNA tumor virus (*a*) because this virus had already been used as a molecular hybridization probe under relaxed conditions of hybridization to screen a variety of malignancies (14, 15), and (*b*) because of the availability of the high-producer JLS-V5 cells, a mouse line which continually secretes MuLV-R

particles. While an extensive relationship between a mouse leukemia virus and a human carcinoma-producing agent would be an unexpected finding, this virus was chosen for preliminary studies on the basis of recent evidence suggesting that a portion of animal oncornavirus genome exhibits an interspecies relationship which closely parallels evolutionary development (16,17). These hybridizations and immunologic tests are therefore predicated on the existence of limited but measurable homology or cross-reactivity between murine and putative human oncornaviruses. Measurements of reverse transcriptase activity, while more direct, utilize reaction mixtures containing divalent cation concentrations which are optimal for known animal viral reverse transcriptase.

Enzyme Studies

Tissue culture fluids from cultured cells derived from needle biopsies of PrCa were examined for reverse transcriptase. Fluids were concentrated by polyethylene-glycol precipitation followed by centrifugation through a discontinuous sucrose gradient selecting for densities between 1:14 and 1:19 g/cm^3. The overall concentration of particulate material of the selected densities was 150-fold. Aliquots of the concentrated material were assayed by a standard DNA polymerase reaction for counts of tritiated thymidine triphosphate incorporated into acid-insoluble product.

Relatively small amounts of polymerase activity insensitive to pretreatment with RNase were detected. This enzyme preferred the synthetic polymer poly(dA) · oligo(dT) over poly(rA) · oligo(dT) (fig 8). This indicates that the DNA polymerase activity was DNA dependent and did not represent reverse transcriptase. Seven cultures of PrCa were tested in this manner with similarly negative results. Under identical conditions murine sarcoma virus (MSV-SD) used as control exhibited at least a fourfold preference for the ribohomopolymer complex, poly(rA) · oligo(dT). It was therefore concluded that if virus or subviral constituents containing reverse transcriptase were being released into the supernatant fluid by these cell cultures, the level of the enzyme was below the detection capability of this assay method.

PrCa tissue has so far been available in too small an amount to be utilized in a search for reverse transcriptase within the cells. One relatively large specimen of BT was homogenized, extracted with high salt and detergent, and chromatographed on DEAE-cellulose. No

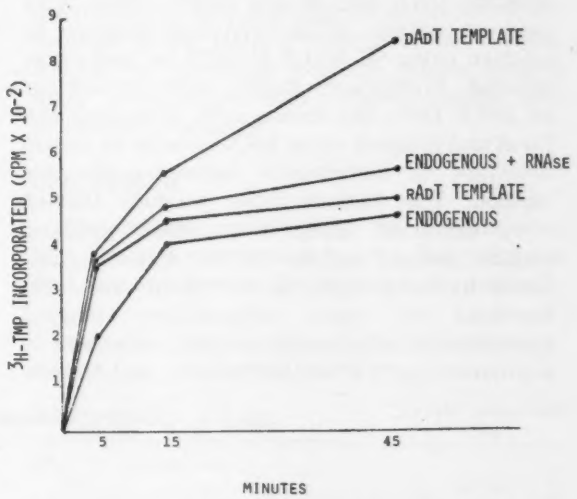

FIGURE 8.—DNA polymerase reaction of a PrCa cell culture. Medium from tissue cultures of these epithelioid cells was pooled and polyethylene-glycol and NaCl were added to 6% and 0.5 M respectively. The pellet from 15-min centrifugation at 2500 × *g* was resuspended in 1/100 of the original volume of TED buffer (100 mM tris [pH 8.3], ethylenediaminetetraacetic acid [1 mM], and dithiothreitol [1 mM]) and centrifuged through 1.14 g/ml sucrose onto 1.19 g/ml sucrose solution (100,000 × *g* for 90 min; SW50.1 Beckman rotor). The opalescent interface was collected and made 0.2% in Triton X-100. A portion was treated with pancreatic RNase (70 μg/ml) for 30 mins at 37°C. 50-μl aliquots were assayed for DNA polymerase activity in a standard 100-μl reaction mixture which contained 5 μmol of tris-HCl (pH 8.3), 0.5 μmol of dithiothreitol, 0.3 μmol of magnesium acetate, 0.08 μmol each of unlabeled dATP, dCTP, and dGTP, and 0.005 μmol of ^{3}H-TTP (50 curies/mmol). The reactions were terminated by the addition of 2.0 ml of cold 5% trichloroacetic acid (TCA) containing 0.02 M sodium pyrophosphate, and 50μg of yeast RNA was added as carrier. After 30 mins the acid-insoluble material was collected on glass fiber filters, washed extensively with 5% TCA, and dried. Radioactivity was determined by liquid scintillation counting using an Omnifluor/toluene cocktail.

DNA polymerase activity which could be identified as reverse transcriptase was found in this specimen.

Molecular Hybridization Studies

Molecular hybridization experiments were carried out in an attempt to determine whether murine oncornavirus-like sequences were present in the DNA of PrCa cells. Normal prostate DNA and several other DNAs were used as controls. RNA-DNA hybridization in solution using ^{3}H-MuLV-R RNA as probe was selected. Preliminary studies were carried out on DNA from the mouse cells producing the virus and progeny virus RNA in order to assure detection of homologous sequences by this method. The various DNA samples studied were extracted using a standard pronase, sodium dodecyl sulfate (SDS) digestion, followed by several phenol-chloroform-cresol extractions at room temperature, ethanol precipitation, alkaline hydrolysis, sonication to a polymer length of 250 nucleotides, and dialysis against distilled water. Viral RNA was obtained from purified MuLV-R virus, lysed by SDS, and subjected to rate zonal centrifugation to obtain high molecular weight RNA. Viral RNA was prepared by Drs. J. L. East and John Knesek of our laboratory. Hybridizations were carried out in 0.24 M phosphate buffer (pH 6.7) at 66°C for varying lengths of time and at several concentrations in sealed capillary pipettes. The percent of viral RNA existing as an RNA-DNA hybrid was assessed by subjecting one half of each sample to pancreatic RNase digestion.

Human placental DNA was initially included as an uninfected control (table 2). It consistently protected 9%–12% of the MuLV-R viral probe, however, so other DNAs were included for comparison; the results are shown in table 3.

An increase in the concentration of JLS-V5 DNA from 1 to 4 mg/ml more than doubled the amount of hybrid formed while an increase of

TABLE 2.—Results of molecular hybridization experiments*

DNA samples	Hybrid formation (%) 48 hrs	168 hrs
Human placenta (4 mg/ml) + MuLV-R	11.6	10.7
JLS-V5 (1 mg/ml)	17.5	17.0
JLS-V5 (4 mg/ml) + MuLV-R	36.9	38.1
Human placenta (4 mg/ml) + RD-114	2.0	4.6
JLS-V5 (1 mg/ml) + RD-114	1.1	1.5
JLS-V5 (4 mg/ml) + RD-114	4.8	5.7

*DNA used in annealing reactions was extracted by homogenization in 1% SDS followed by the addition of pronase to 1 mg/ml and incubation at 37°C for 16–18 hrs. An equal volume of a mixture of phenol-chloroform-cresol (1:1:0.15), containing 1/20 volume of 2 M tris (pH 9.0), was added and the mixture was shaken at room temperature and centrifuged to phase separation. Usually 3 such extractions resulted in a clear organic phase. The aqueous phase was then precipitated with 2.5 vols of ethanol and 0.5 vols of 4 M NaCl. After resuspension of the pellet in TED buffer the solution was sonicated in a Branson sonifier sufficiently to reduce DNA molecules to an average chain length of 250 nucleotides. Sodium hydroxide was added to 200 mM final concentration and the mixture was incubated at 37°C for 30 mins. Dialysis against 5× TED buffer for 30 mins was followed by dialysis against distilled water for 24 hrs (2 × 100 vols). DNA determinations were made spectrophotometrically. Viral RNA preparations were prepared by East et al (18) as previously described. Hybridizations were performed as described by East et al (19) with the substitution of 0.24 M sodium phosphate buffer as the 2× buffer.

TABLE 3.—Results of molecular hybridization experiments using other DNAs for comparison*

DNA samples (mg/ml)	Hybrid formation (%)
Calf thymus	
15	11.9
30	10.6
60	11.7
E. coli	
15	2.4
30	3.1
60	5.9
Salmon testis	
30	6.5
60	9.7
Human placenta	
60	12.4
JLS-V5	
30	52.0
60	63.5

*See footnote to table 2.

the annealing time gave little increase in hybrid formation (table 2). RD-114 virus RNA was included for comparison as a control. It may be seen (table 2) that RD-114 RNA was not well protected by sequences in human placental or JLS–V5 DNA.

Increase of the JLS-V5 DNA concentration increased the percent of hybrid formation with MuLV-R RNA as shown in table 4. This did not take place with the RNA of RD-114 or when the concentration of human placental DNA was increased for MuLV-R or RD-114 probes.

It was found that calf thymus DNA gave a degree of protection which did not increase with increasing DNA concentration (table 3). *Escherichia coli* and salmon testis DNA gave lower values. Human placental DNA consistently gave 9%–12% of hybrid formation and JLS-V5 gave the maximum percent (63%). These results indicate that a high degree of homology can be demonstrated to exist between MuLV-R and its producer cell DNA. The meaning of the small percent of homology between human placental, calf thymus, and salmon testis DNA and MuLV-R RNA is under study.

When normal prostate DNA and PrCa DNA were tested with labeled MuLV-R RNA, no hybrid formation resulted at 48 hours and very low amounts were obtained at 144 hours (table 5). The DNA concentration used was 4 mg/ml.

The results of these preliminary hybridization studies indicate that the molecular hybridization method can detect the presence of MuLV-R-specific gene sequences in the DNA of cells shown to carry the virus. Under the same conditions no MuLV-R-specific sequences were detected in the DNA of cells of either normal, benign, or cancerous prostate. These studies must be continued with different probes and additional specimens.

DISCUSSION

The evidence provided by the results of a number of experimental approaches supports the idea that viruses may be etiologically

TABLE 4.—Effect of DNA concentration (48-hr hybridization)*

DNA samples (mg/ml)	Hybrid formation (%)
JLS-V5 + MuLV-R	
5.6	44.1
11.2	53.4
14.4	60.7
JLS-V5 + RD-114	
14.4	7.4
Human placenta + MuLV-R	
4	9.0
16	9.4
Human placenta + RD-114	
16	7.6

*See footnote to table 2.

TABLE 5.—Normal and carcinomatous prostatic DNA tested with labeled MuLV-R*

	Hybridization (%)	
DNA samples†	48 hrs	144 hrs
Normal prostate + MuLV-R	0	2.5
Prostatic carcinoma + MuLV-R	0	3.0
Human placenta + MuLV-R	6.3	7.9
JLS-V5 + MuLV-R	32.2	36.8

*See footnote to table 2.
†All DNA concentrations are 4 mg/ml.

associated with human neoplasia of various types. This viral association, particularly with breast carcinoma, nasopharyngeal carcinoma, and cervical carcinoma, adds particular interest to the possibility that viruses may also play a role in the origin of human PrCa.

The studies described in this paper have been initiated in an attempt to obtain morphologic, immunologic, biologic, and biochemical data implicating either oncornaviruses or herpesviruses (or both) in PrCa in man. The results, though still quite preliminary, indicate the presence of structures resembling type-C virus particles in tissue from both PrCa and BPH.

Antibodies to a variety of as yet uncharacterized antigens in prostatic tissues can be demonstrated in the sera of patients with PrCa. Some of these antigens may be viral, as indicated by tests with heterologous antiviral sera. It should be noted that some of these data were obtained with MA-160 cells, reportedly derived from human prostatic tissue but whose identity must be verified. In any case, the clear differences between reactions of sera from PrCa patients and those from normal donors, as shown by the results of tests employed, are of interest and must be further investigated. No definite conclusions can be drawn from the results of molecular hybridization studies at this early stage. The techniques are applicable to these studies, however, and will be continued with more prostatic material and different viral probes. As viewed from this early stage of investigation, the search for viruses in human PrCa by the methods outlined appears feasible and promising.

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Virology of Prostatic Cancer [1]

Edgar J. Sanford, Thomas J. Rohner, and Fred Rapp [2,3]

The etiology of prostatic carcinoma will most likely be found to include multiple factors. Fragmentary evidence exists from human epidemiologic data that prostatic tumors are, or can be, influenced by race, age, and perhaps socio-economic factors. It has been known for years that prostatic cancer is markedly endocrine responsive. Prostatic tumors can be induced in in vivo and in vitro animal systems by chemical carcinogens, viruses, hormones, and combinations of these agents. Can a common ground be found to link known oncogenic processes together? Tumor production infers a heritable change, presumably at the level of nucleic acids, which results in redirected cellular processes in such a manner to produce cancer. Complete and defective viruses have the property of introducing nucleic acids into cellular genomes and would therefore seem to be prime candidates for the ultimate common ground.

Man, canines (1), and hamsters (2) are the only known species in whom prostatic carcinomas spontaneously arise in vivo. Because castration completely eliminated the spontaneous development of prostatic adenocarcinoma in the Syrian golden hamster, Fortner (2) felt that endocrine stimulation could be an important factor in spontaneously arising prostatic carcinoma. The rarity of prostatic tumors in dogs has severely limited oncogenesis research in this species. Observations concerning spontaneously arising prostatic cancer in man will be reviewed in this paper.

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[2] Division of Urology, Department of Surgery (Drs. Sanford and Rohner), and Department of Microbiology (Dr. Rapp), M. S. Hershey Medical Center, The Pennsylvania State University College of Medicine, Hershey.

[3] *Reprint requests to*: Edgar J. Sanford, MD, Division of Urology, Department of Surgery, M. S. Hershey Medical Center, Pennsylvania State University College of Medicine, Hershey, Pa 17033.

Successful attempts at experimentally producing carcinoma of the prostate in vivo have been limited primarily to chemical carcinogens. Prostatic tumors in mice (3), rats (4), and hamsters (5) have been produced by various chemical carcinogens. Varkarakis and his colleagues (6) using combinations of 7,12-dimethylbenzanthracene (DMBA), adenovirus type 12, estradiol, and testosterone found no detectable carcinogenic effect unless either the virus or the chemical carcinogen were combined with a hormone. Adenovirus type 12 injected directly into the prostate of intact or castrated hamsters did not produce tumors unless combined with estradiol. These tumors were sarcomatous in type and did not show any of the principal characteristics of human prostatic adenocarcinoma. Thus, production of an in vivo animal prostatic adenocarcinoma model similar to that occurring naturally in man has eluded investigators.

In vitro cell experiments have been more successful. Paulson et al (7) described the transformation of hamster prostate tissue in vitro with SV40. Fraley and Paulson (8) demonstrated that the SV40-transformed prostatic tissue when injected into hamsters exhibited progressive growth leading to the death of the animal. These tumors were shown to be both estrogen and androgen dependent and to produce an elevated serum acid phosphatase level. Unlike human prostatic cancer, these tumors did not form glands nor produce osseous metastases. Though virus-specific T antigen could be readily identified by immunofluorescence, no virus particles could be recovered from the cultured tumor cells. Abdalla and Oliver (9) confirmed this work but failed to see any androgen effect on tumor growth. Though there is no report of virus transformation of human prostatic tissue, Fraley and his colleagues (10, 11) developed a spontaneously transformed human prostate line (MA 160) from a benign prostatic adenoma. Viruses could

not be identified in MA 160, either by electron microscopy, co-cultivation experiments, or inoculation of a variety of human cell lines with cell-free filtrates. Further in vitro work with other complete viruses or defective particles is needed. Of special interest would be the attempted transformation of human prostatic tissue with irradiated herpesviruses, as has been done with hamster embryo fibroblasts and herpes simplex virus types 1 and 2 (12–14).

What is the evidence for a relationship between adenocarcinoma of the prostate and viruses in man? Though fragmentary, present data continue to point at viruses. The difficulty of growing human prostatic cancer in cell culture has been a limiting factor in these investigations. Röhl (15) first described the growth of human prostatic carcinoma from explant on plasma clots in 1959. Stonington and Hemmingsen (16) described an explant technique in 1971. Successful growth of 21 human prostatic adenocarcinomas, again using an explant technique, has also been reported (17). Tissue was obtained at total perineal prostatectomy, but tumor from needle biopsies and transurethral resections was also used. The in vitro lifetime of these cultures did not exceed 2 or 3 months. Brehmer and his colleagues (18) established 105 primary cultures from the prostatic tissue of 22 patients. Sixty-one were established from explants and 44 were started from cell suspensions following trypsinization. Of these, 18 primary suspension cultures and 27 explant cultures were established from prostatic carcinomas. Growth occurred in all cases; however, viability as noted above could only be maintained for 2 or 3 months (ten to 12 passages). It was noted that cells obtained from adenocarcinoma showed a higher plating efficiency than those obtained from hyperplasia.

Evidence for virus particles in prostatic fluid has been conflicting. The etiology of common nonspecific prostatitis is unclear. Morrisseau et al (19) studied prostatic fluid obtained by massage from 12 patients with prostatitis and ten asymptomatic controls. Inoculates were made into human diploid fibroblasts (WI–38) and human embryonic kidney cells. No viral agents were isolated from the controls. Two of the 12 prostatitis patients showed cytopathic effects suggestive of herpes simplex virus. Centifanto et al (20) studied prostatic secretions in 190 randomly selected men and found herpes simplex virus in 15%. These investigators felt that the prostate may serve as a reservoir for the venereal transmission of this virus. However, other investigators (21) failed to uncover evidence of any type of virus in 50 samples from patients with prostato-urethritis, nor could they find an increased incidence of humoral antibody against herpes simplex virus type 2 in affected patients compared with matched asymptomatic controls. Likewise, Nielsen and Vestergaard (22) failed to isolate any virus from postprostatic massage urine specimens or prostatic tissue obtained from needle biopsies in 48 patients. All the above investigators were able to isolate herpes simplex virus directly from genital herpetic lesions. A possible explanation for the discrepancy may be in collection methods. Both Fabricant (23) and Weller and Hanshaw (24) found that storage of viruses in urine prior to culturing would inactivate human cytomegalovirus (a herpesvirus).

Electron microscopic examination of human prostatic carcinoma has also yielded findings suggestive of a tumor-associated virus. Tannenbaum et al (25, 26) have noted virus-like particles of the same configuration and measurements as the Lucké tumor virus (a herpesvirus). These consisted of elongated, thread-like particles found in normal cells around the cancerous areas in 48 of 61 specimens studied and directly in the tumor cells of eight of the 61 specimens. In addition, these particles were seen in one of three human semen specimens examined. These same investigators noted identical particles in human breast cancers, both inside the tumor cells and in the secretion of peripheral cells around them. Brehmer and his associates (27) noted similar particles close to the cell surface in human prostatic cancer cells grown in cell culture. Examination of human prostatic cancer grown in cell culture has also revealed intranuclear inclusions characteristic of herpes-type viruses by light microscopy (28). Electron microscopy then confirmed the presence of virus-like particles within the nuclei of the tumor cells. The size and configuration of

these particles were consistent with the presence of a herpes-like virus. Using monolayer cell culture from one patient with carcinoma of the prostate, Centifanto and colleagues (29) demonstrated the presence of herpesvirus antigens in the nuclei of tumor cells by specific immunofluorescent staining. These investigators emphasized the fact that these herpesvirus antigens were found in tissue explants and not in repeatedly passed cell cultures. No lytic or cytopathic effects were observed. They felt that this finding was comparable to that of Frenkel et al (30) who found a herpes simplex DNA fragment but no infectious virus in cervical cancer cells taken from one patient. Intranuclear inclusions were also seen in sections taken directly from the original tumor by electron microscopy. Using another approach, Farnsworth (31) found reverse transcriptase activity, indicative of the presence of an RNA tumor virus, in human prostatic tissue. Significant enzyme activity was greatest in a patient with prostatic cancer but was also present in histologically benign specimens.

Viral epidemiologic studies of human prostatic carcinoma are even more scant but suggestive. Shah and co-workers (32) failed to find a significantly increased titer of SV40–neutralizing antibodies in the sera of patients with bladder and prostatic carcinomas when compared with matched controls. Ravich (33) reported from his own practice a 20% incidence of prostatic carcinoma in largely uncircumcised Caucasian non-Jews compared to only 1.7% for Jews. Other socio-economic factors being equal, it was felt that the only significant difference between these two groups was the presence or absence of the prepuce. The author felt that these facts might indicate the association of a venereally transmitted virus infection with prostatic carcinoma. Lattimer's epidemiologic survey of 101 prostatic cancer patient's wives disclosed an increased incidence of genital organ cancer which was statistically significant (34). Indeed, the greatly diverse incidence rates between races and geographic areas are difficult to interpret in terms of environmental factors (35).

More attention has been focused on implicating herpesviruses in other genital tumors by the findings of Hollinshead and Tarro (36) of antibody for herpesvirus nonvirion antigen in human lip and cervical carcinomas and the demonstration of antibody to herpes simplex virus type 2-induced tumor-specific antigens in sera from patients with cervical carcinoma (37). Most significantly, Sabin and Tarro (38) found antibodies against a presumed herpesvirus-specified antigen in the sera in eight of eight patients with carcinoma of the prostate. A similar correlation was found in nine other tumor types but a similar antibody was not found in patients without cancer nor in patients with 21 other types of malignancies.

Studies with herpesviruses are further complicated by the fact that irradiation (fig 1) or photodynamic inactivation (fig 2) of the virus yields preparations that can transform cells in vitro (figs 3–5) into a malignant phenotype (12–14). Metastases often occur after reimplantation of the cells into the original species. These results (ie, transformation in vitro) have been extended to mouse (39,40), rat (41,42),

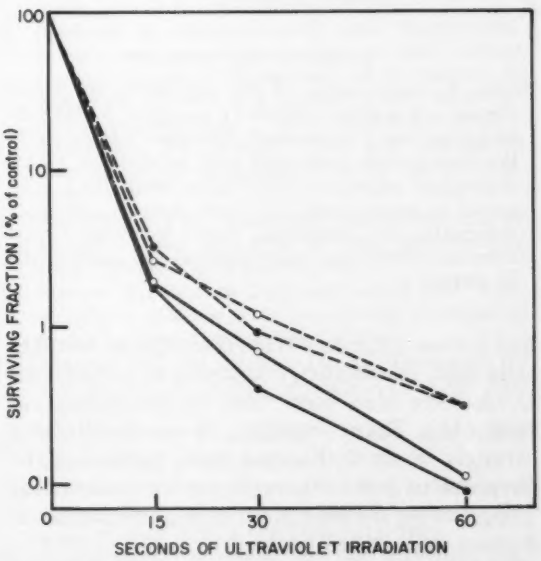

FIGURE 1.—Inactivation of the infectivity of herpes simplex virus type 1 (○) and type 2 (●) by exposure to ultraviolet light. Virus inactivated past detectable levels can still transform mammalian cells in vitro.

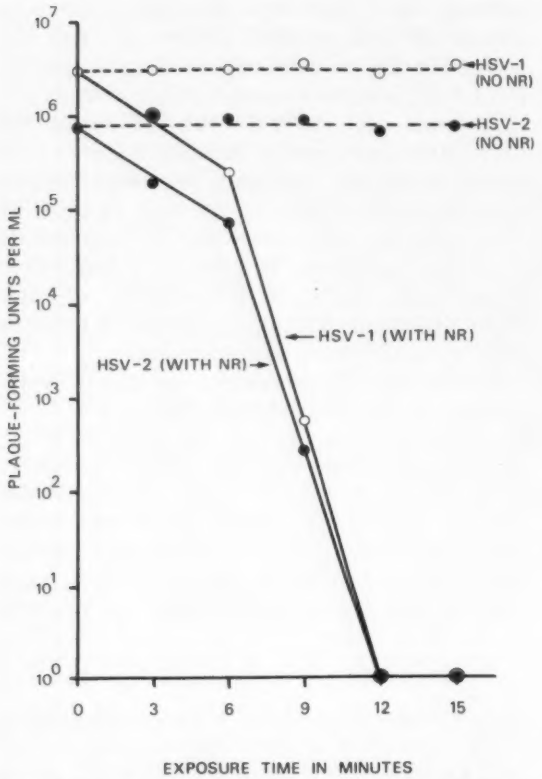

FIGURE 2.—Inactivation of the infectivity of herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) by photodynamic procedures. The virus was grown in the dark in cells pretreated with neutral red (NR). Subsequent exposure of the virus (with DNA complexed to neutral red) to visible light caused rapid destruction of infectivity. Virus inactivated past detectable levels can still transform mammalian cells in vitro.

and human (43) cells from the original hamster cells used. In addition, fragments of herpesvirus DNA have also been used to transform rat cells (44). Taken together, these observations strongly imply that only a small portion of the herpesvirus genome is required for transformation, making the search for foreign materials in human cells more difficult.

Another feature incriminating herpesviruses as oncogenic agents is stimulation of host DNA biosynthesis by human cytomegalovirus strains (45). Again, this virus has been shown to transform cells in vitro (46).

FIGURE 3.—Normal hamster embryo fibroblasts growing in cell culture (stained with hematoxylin and eosin).

FIGURE 4.—Hamster embryo fibroblasts transformed by inactivated herpes simplex virus. The cells are primarily fibroblastic and induce a fibrosarcoma when inoculated into hamsters.

What is in the future? Circumstantial evidence connecting viruses with prostatic neoplasia cannot be ignored. Further work towards detecting virus genomes within cancer cells using serologic or biochemical techniques should

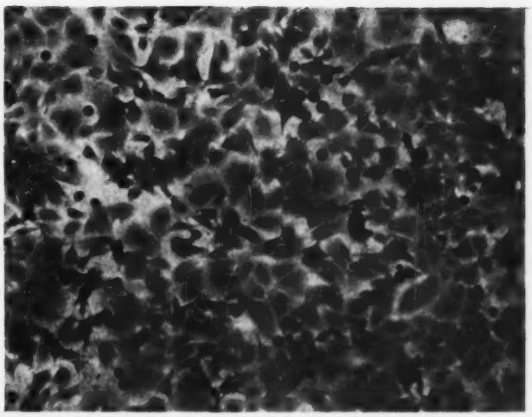

FIGURE 5.—Hamster embryo fibroblasts transformed by inactivated herpes simplex virus. The cells are primarily epithelioid and induce an adenocarcinoma when inoculated into hamsters.

be carried out. Virology of prostatic carcinoma remains in its infancy but continues to yield hopes for greater understanding and ultimate control of this neoplasm.

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The Search for "Virogene" in Human Prostatic Tissues: Prostatic DNA Polymerases [1]

S. K. Arya, W. A. Carter, R. F. Zeigel, and J. S. Horoszewicz [2,3,4]

SUMMARY

The "virogene-oncogene" hypothesis of Huebner and Todaro and the "provirus" hypothesis of Temin implicate RNA tumor viruses in the neoplastic transformation of mammalian cells. These hypotheses have been substantiated in several animal systems including primates and, presumably, in man. Because the detection in a tissue of one or two activities allegedly related to RNA tumor virus may not be conclusive evidence for viral presence, we have developed a scheme of coordinated morphologic, biologic, and biochemical investigations of human prostatic tissues. We report here the more recent progress we have made in one of the segments of our scheme of investigations. Two, possibly three, DNA polymerase activities from human prostatic tissue have been isolated and partially purified by DEAE-cellulose and phosphocellulose chromatography. These activities have been partially characterized. Based on template preferences and non-inhibition by selective inhibitors of reverse transcriptase, neither of the major polymerase activities appears to be the reverse transcriptase-type activity.

[Cancer Chemother Rep 59:39–46, 1975]

The "virogene-oncogene" hypothesis formulated by Huebner and Todaro (1,2) postulates that the genetic information of RNA tumor viruses (called "virogene") and a portion (or whole) of "virogene" responsible for malignant transformation (called "oncogene") are present in normal mammalian cells but in an unexpressed or repressed state. These genes may be activated by a variety of agents, such as radiation, chemical carcinogens, "aging," hormonal influences, and exogenous viruses. The hypothesis suggests that the cells containing the "virogene" information may undergo neoplastic transformation by activation of the "oncogene." It does not require the expression of the whole "virogene" leading to the production of mature virus particles. The activation of the "oncogene" may be sufficient for the eventual emergence of transformed cells. Thus, neoplastic cells may contain mature virions, subviral components (eg, viral genome), and their expressed functions (eg, gs antigens).

The "provirus" hypothesis of Temin (3) states that the neoplastic transformation of

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[2] Department of Medical Viral Oncology, Roswell Park Memorial Institute (RPMI), and the Graduate Faculty in Microbiology, Roswell Park Division, State University of New York, Buffalo.

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[4] *Reprint requests to*: Dr. S. K. Arya, Department of Medical Viral Oncology, RPMI, 666 Elm St, Buffalo, NY 14203.

cells by RNA tumor viruses is accompanied by the integration of viral genomic information into the host cell genome. This hypothesis requires a mechanism for the conversion of viral genetic information present as RNA into a DNA copy or "provirus." The discovery by Baltimore (4) and Temin and Mizutani (5) of a novel RNA-directed DNA polymerase, otherwise known as reverse transcriptase, associated with RNA tumor viruses provided a strong support for the "provirus" hypothesis. The reverse transcriptase activity has been demonstrated in several mammalian cells infected or transformed by RNA tumor viruses (6,7). Such enzymatic activity has been recently shown to be present in human neoplastic cells and tissues. For example, Gallo and his co-workers have presented evidence demonstrating reverse transcriptase activity in human leukemic lymphocytes (8,9) and Spiegelman and coworkers (10,11) have shown the same in human lymphomas and breast carcinoma. Thus, a demonstration of reverse transcriptase-type activity in human neoplastic tissues may serve as an indicator for the presence of virus or its expressed functions.

It is recognized that the detection of one or two activities allegedly related to RNA tumor viruses may not provide conclusive evidence for the presence of, and transformation by, these viruses. We have, therefore, decided on a scheme of coordinated and correlated investigations which attempts to examine the human prostatic tissue morphologically, biologically, and biochemically. It is designed to detect and correlate the presence of mature virions and several viral functions in normal, hyperplastic, and malignant prostatic tissue, and to investigate the factors which modulate the viral activities in these tissues.

Scheme of Investigations

A brief outline of the investigation scheme is presented in figure 1. The scheme consists of three major segments: (*a*) ultrastructural, (*b*) cell biologic, and (*c*) biochemical studies. The ultrastructural studies concentrate initially on the visualization of mature virions and nucleocapsids and examine the effect of the presence of virus on intracellular and intercellular organization by light and electron microscopy. The cell biologic studies attempt to obtain short-term cultures of prostatic tissue and to score for the presence of virus by manipulation of the culture conditions (ie, variations in the pH of the medium and exposure to iododeoxyuridine or cyclohexamide, treatments known to activate endogenous viruses [12–15]). The effect of antiviral agents (eg, streptovaricins, interferon, and modified polynucleotides) forms an integral part of these studies. The antiviral properties of streptovaricins (16) [5] and interferon (17,18) are well established. We have also been studying the synthetic polynucleotides as antiviral agents (19) and selective inhibitors of viral reverse transcriptase (20). An application of these polynucleotides as diagnostic tools for distinguishing viral DNA polymerase from cellular DNA polymerase is described below. The cell biologic studies will also include the cultivation of the prostatic tissue extracts with virus-permissive mammalian cells and will be extended to the study of somatic cell hybrids of prostatic and other mammalian cells.

The biochemical studies encompass two major subsegments. One involves the detection of the viral genetic information integrated in the prostatic DNA and the determination of the number of copies and nature of the site of integration of viral genome in the prostatic genome by molecular hybridization techniques. The second segment involves the isolation, characterization, and study of the enzymatic properties of DNA polymerases, particularly reverse transcriptase, from prostatic tissues of normal, hyperplastic, and malignant origins. The latter also includes the physicochemical characterization of the virions and endogenous polymerase activity.

Initially, we morphologically examined some 20 specimens and biochemically examined one to five specimens obtained by transurethral biopsy. No evidence of the presence of virus particles or reverse transcriptase was obtained in these specimens. Because of the sparse content of epithelial cells received in such specimens and otherwise insufficient tissue

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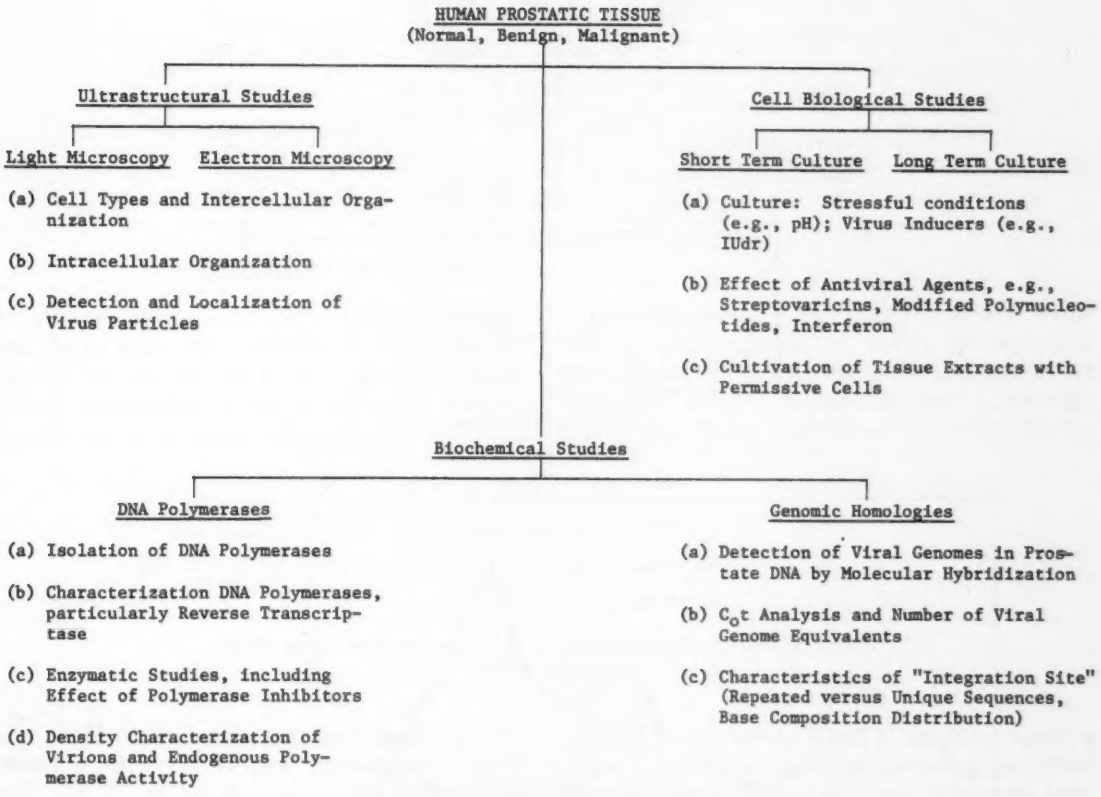
HUMAN PROSTATIC TISSUE
(Normal, Benign, Malignant)
Ultrastructural Studies
Light Microscopy
Electron Microscopy
(a) Cell Types and Intercellular Organization
(b) Intracellular Organization
(c) Detection and Localization of Virus Particles
Cell Biological Studies
Short Term Culture
Long Term Culture
(a) Culture: Stressful conditions (e.g., pH); Virus Inducers (e.g., IUdr)
(b) Effect of Antiviral Agents, e.g., Streptovaricins, Modified Polynucleotides, Interferon
(c) Cultivation of Tissue Extracts with Permissive Cells
Biochemical Studies
DNA Polymerases
(a) Isolation of DNA Polymerases
(b) Characterization DNA Polymerases, particularly Reverse Transcriptase
(c) Enzymatic Studies, including Effect of Polymerase Inhibitors
(d) Density Characterization of Virions and Endogenous Polymerase Activity
Genomic Homologies
(a) Detection of Viral Genomes in Prostate DNA by Molecular Hybridization
(b) C_0t Analysis and Number of Viral Genome Equivalents
(c) Characteristics of "Integration Site" (Repeated versus Unique Sequences, Base Composition Distribution)

FIGURE 1.—Scheme of coordinated investigations of human prostatic tissue.

material for detailed biochemical studies, we decided to investigate fresh autopsy tissue. We felt it prudent to examine first the normal and hyperplastic tissues to establish baselines for the several parameters we wish to measure. We briefly review here some of the recent progress we have made in one of the segments of our investigation scheme, namely, the isolation and characterization of the DNA polymerases from human prostatic tissues.

DNA Polymerases of Prostatic Tissue

An outline of the scheme used for isolation of DNA polymerases is shown in figure 2. This scheme is based on that used successfully by Gallo and his co-workers (9,21,22) for showing the differences between DNA polymerases from normal and leukemic human lymphocytes. The noteworthy features of this procedure are the removal of lysosomes before the lysosomal enzymes can cause extensive damage to the polymerases and the production of the high-speed supernatant containing solubilized polymerase activities which are then purified by successive chromatography on DEAE-cellulose and phosphocellulose.

The DEAE-cellulose chromatography of the high-speed supernatant (S-120, fig 2) from a hypertrophic prostatic tissue specimen (PR A-74-81) is shown in figure 3. The supernatant (800 mg of protein) containing the solubilized polymerase activity was applied to a DEAE-cellulose column (2.6 × 26 cm) equilibrated with TEMG (0.05 M tris-HCl [pH 7.8], 0.001 M EDTA, 0.001 M dithiothreitol, and 20% [vol/vol] glycerol). The column was developed with

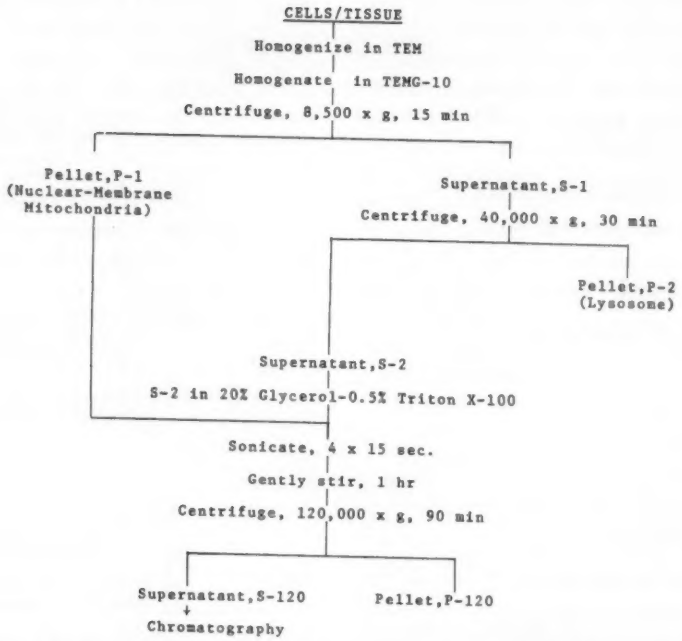

FIGURE 2.—Scheme for isolation of DNA polymerases from human prostatic tissue.

a linear gradient of NaCl (0–0.6 M) in TEMG, collecting fractions in tubes containing a sufficient amount of dialyzed bovine serum albumin (BSA) to make the fraction 0.5 mg/ml in BSA. The fractions were assayed for polymerase activity using poly[d(A-T)] as a template. The chromatogram shows a broad peak of polymerase activity eluting at about 0.24 M NaCl with shoulders at 0.18 and 0.28 M NaCl. There is an additional small peak of activity eluting at about 0.35 M NaCl (fig 3). The observed resolution of activities is somewhat unexpected. In general, DEAE-cellulose chromatography is believed to result in the separation of nucleic acids from polymerases (an important step for further characterization of polymerases) and not in the resolution of polymerase activities which may be present in the tissue extract.

The fractions containing the polymerase activities were pooled, concentrated with Ficoll, and dialyzed against TEMG-0.1 M NaCl. The resulting solution (156 mg of protein including BSA) was applied to a phosphocellulose column (1.5 × 15 cm) equilibrated with TEMG-0.1 M NaCl. The column was eluted with a linear gradient of NaCl (0.1–0.6 M) in TEMG. The fractions, collected in tubes containing enough BSA to make 0.5 mg/ml, were assayed for polymerase activity using poly[d(A–T)], activated DNA, and poly(A) · oligo(dT) as templates (fig 4). When the assays were performed immediately after developing the column using poly[d(A–T)] as template, three peaks of polymerase activity centered at 0.32, 0.40, and 0.45 M NaCl were observed. We have tentatively termed these activities polymerase α, β, and γ respectively, although it has not yet been ruled out that one or more of these activities may contain terminal nucleotidyl transferase. The estimated purification achieved for these activities is summarized in table 1. The assays using activated DNA and poly(A) · oligo(dT) as templates, performed after storage of the fractions at 4°C for 3–5 days, did not show polymerase activity corresponding to the polymerase α. Reassaying of the fractions with poly[d(A-T)] at this time also failed to score

TABLE 1.—Purification of DNA polymerases from human prostate

Fraction	Protein (mg)	Activity* (pmol/mg)	Relative activity	% Yield (protein)
Extract, S-120	800	0.44	1	100
DEAE pool	46	11.2	25.4	5.75
Phosphocellulose				
Pool α	3	27.5	62.5	0.37
Pool β	2	400	909	0.25
Pool γ	2	600	1363	0.25

*Incorporation of TMP in 30 mins at 37°C with poly[d(A-T)] as template (*see* legend to fig 3).

for this activity. These observations suggest that the polymerase α activity is labile and is rapidly lost on storage at 4°C. There is some loss of polymerase β and γ activities during storage but it is not as dramatic.

The polymerase β and γ activities are being further characterized in terms of template preferences, molecular weights, salt-dependent molecular weight transitions, and enzymatic properties. These studies will provide the in-

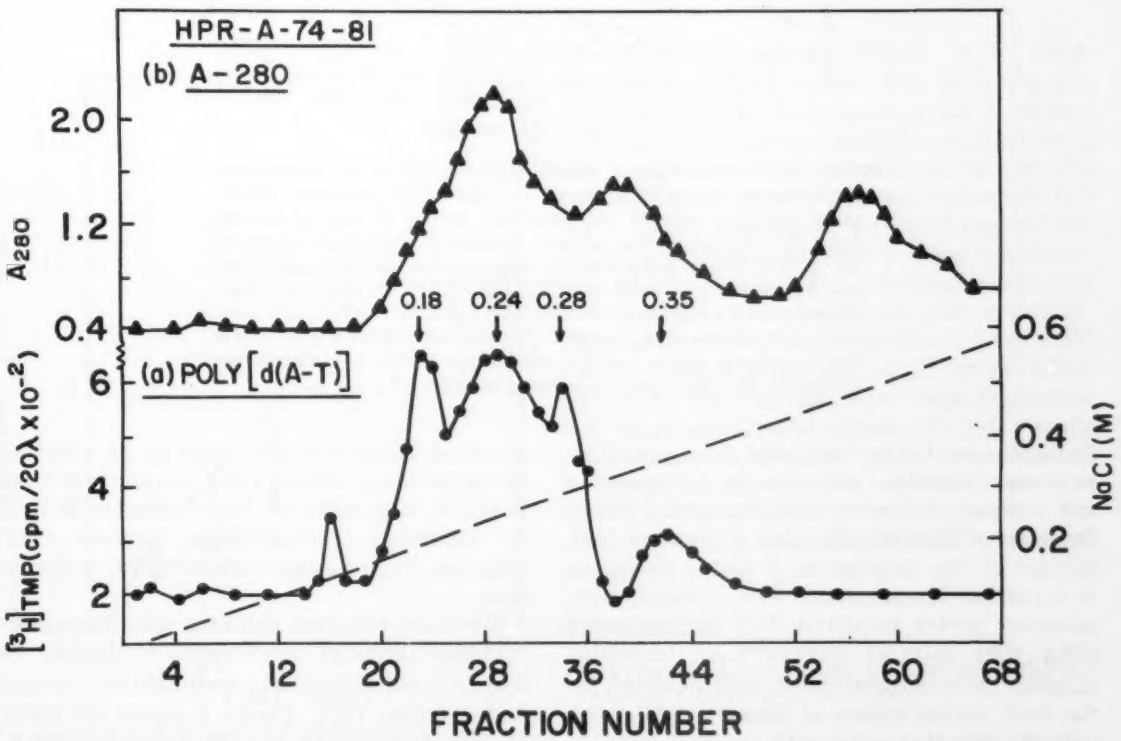

FIGURE 3.—DEAE-cellulose chromatography of DNA polymerase activity of human prostatic tissue extract. (*a*) Polymerase activity was assayed in a reaction mix composed of 0.05 M tris-HCl (pH 7.9), 0.02 M dithiothreitol, 0.006 M $MgCl_2$, 100 μM poly[d(A-T)], 100 μM dATP, 10 μM [^{3}H]dTTP (4000 cpm/pmol), and aliquots from column fractions. The reaction mix was incubated at 37°C for 30 mins and aliquots were precipitated with trichloroacetic acid and counted. (*b*) Absorbance at 280 mμ including that contributed by added BSA (0.5 mg/ml).

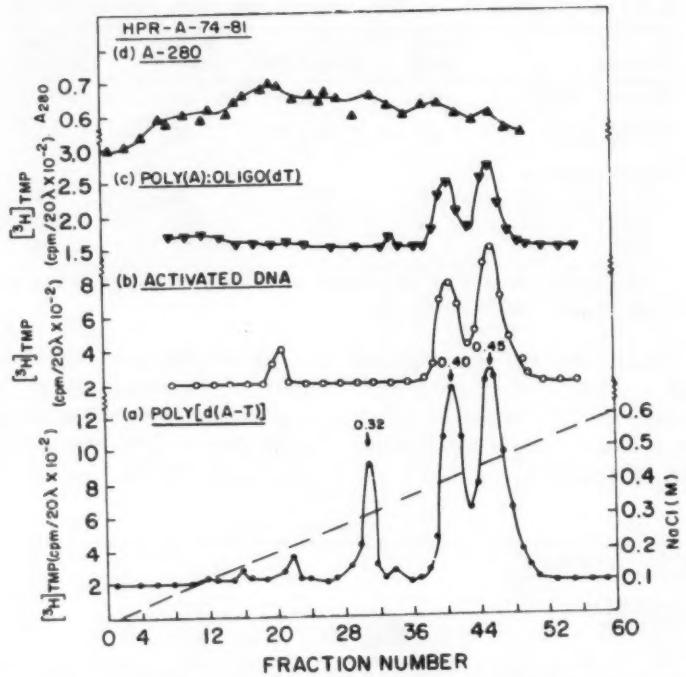

FIGURE 4.—Phosphocellulose chromatography of DNA polymerases from human prostatic tissue. (*a*) Poly[d(A-T)]-directed polymerase activity, assayed as described in fig 3. (*b*) Activated DNA-directed polymerase activity, assayed in a reaction mix similar to (*a*), where poly[d(A-T)] was replaced by activated DNA and included 100 μM each of dGTP and dCTP. (*c*) Poly(A)·oligo-(dT)-directed polymerase activity, assayed in a reaction mix similar to (*a*), where poly(A)·oligo(dT) replaced poly[d(A-T)], 0.02 M $MnCl_2$ replaced $MgCl_2$, and did not include dATP. (*d*) Absorbance at 280 mμ including that contributed by BSA (0.5 mg/ml).

formation needed to designate these activities as normal cellular polymerases (cytoplasmic and nuclear) and reverse transcriptase. From the results obtained thus far, it appears that neither of the polymerase β and γ activities is a reverse transcriptase. For example, both activities prefer poly[d(A–T)] and activated DNA over poly(A)·oligo(dT) as templates (23,24). This suggestion is substantiated by the lack of inhibition of these activities by poly(2′-0-ethyl)adenylic acid (*see* below).

As mentioned above, we have been investigating the antiviral and anti-reverse transcriptase properties of synthetic polynucleotides. One of the aims of these studies is to develop reagents which will inhibit the reverse transcriptase selectively with little or no effect on the activities of cellular DNA polymerases. Such reagents may serve as useful diagnostic tools in ultimately distinguishing reverse transcriptase from normal cellular DNA polymerases.

We have obtained evidence which suggests that 2′-0-alkylated polyadenylic acids may be selective inhibitors of oncornaviral reverse transcriptase (20). Figure 5 shows the effect of polyadenylic acid, poly(2′-0-methyl)adenylic acid, and poly(2′-0-ethyl)adenylic acid on the DNA polymerase activities from Moloney murine leukemia virus (MLV), *Escherichia coli*, and mouse spleen. It is evident that these polyadenylic acids, especially poly(2′-0-ethyl)-

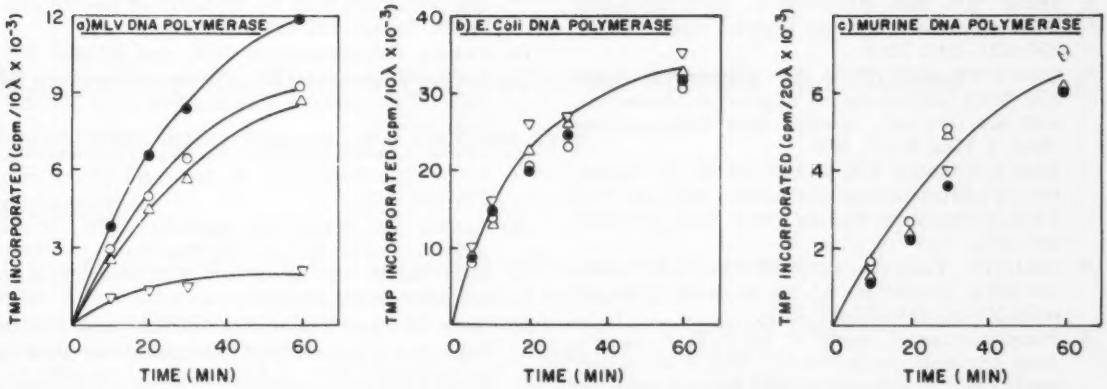
a) MLV DNA POLYMERASE
b) E. Coli DNA POLYMERASE
c) MURINE DNA POLYMERASE
TMP INCORPORATED (cpm/10λ X 10⁻³)
TMP INCORPORATED (cpm/20λ X 10⁻³)
TIME (MIN)

FIGURE 5.—Effect of polyadenylic acids on DNA polymerase activity from (*a*) MLV, (*b*) *E. coli*, and (*c*) mouse spleen. The reaction mix contained 0.05 M tris-HCl (pH 7.9), 0.06 M NaCl, 0.006 M $MgCl_2$, 0.02 M dithiothreitol, 100 μM dATP, 50 μM [^{3}H]dTTP (2600 cpm/pmol), 50 μM poly[d(A-T)], and appropriate enzyme preparation: -●-●- = poly[d(A-T)]; -○-○- = poly[d(A-T)] plus poly(A) (10 μM); -△-△- = poly[d(A-T)] plus poly(2′-0-methyl)adenylic acid (10 μM); -▽-▽- = poly[d(A-T)] plus poly(2′-0-ethyl)adenylic acid (10 μM).

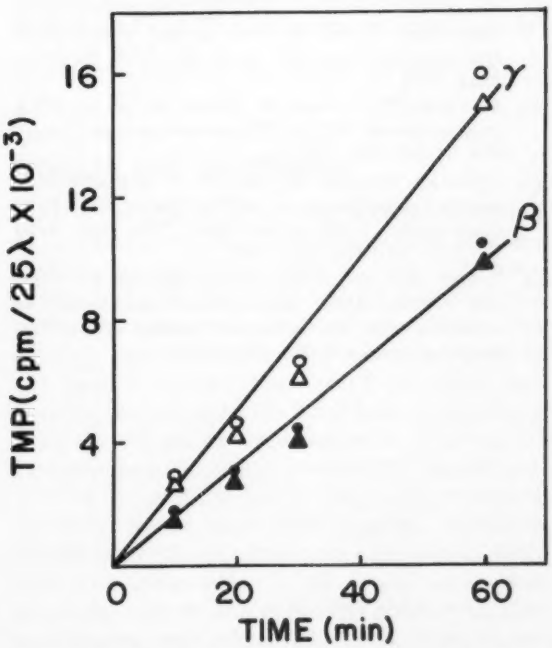

FIGURE 6.—Effect of poly(2′-0-ethyl)adenylic acid on DNA polymerases from human prostatic tissue. The reaction conditions were the same as described in fig 5. Polymerase β activity in the absence (-●-●-) and presence (-▲-▲-) of poly(2′-0-ethyl)adenylic acid. Polymerase γ activity in the absence (-○-○-) and presence (-△-△-) of poly(2′-0-ethyl)adenylic acid.

adenylic acid, strongly inhibit MLV DNA polymerase without significantly affecting the activities of the two cellular enzymes. As shown in figure 6, the human prostatic polymerase β and γ activities are not inhibited by poly(2′-0-ethyl)adenylic acid. These results suggest that the prostatic polymerase activities are cellular DNA polymerases and not reverse transcriptase of viral origin. The results also reaffirm and strengthen the evidence that poly(2′-0-ethyl)adenylic acid is a selective inhibitor of reverse transcriptase with little or no effect on cellular DNA polymerases. These studies are now being extended to human prostatic tissues of different morphologic types, from normal to frankly malignant. The results of our morphologic, biologic, and other biochemical investigations of prostatic tissues will be reported elsewhere.

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Human Prostatic Tumors in Conditioned Animals and Culture

Gordon Sato, Walter Desmond, and Francoise Kelly [1,2]

SUMMARY

A nude mouse colony has been established with a capacity of over 1000 mice. These mice have been injected with human prostatic tumors. A spleen injection method has been developed which enables us to follow the growth of tumor cells in the animal for short periods of time and to assess the effects of hormones on this growth. We have also studied the behavior of several animal cell lines in culture and in nude mice as possible models for a hormone-dependent human prostatic tumor. By using nude mice, we have been able to show that apparent "normal" revertants of cancer cells are actually antigenic variants which can be used to immunize animals against the original tumor cells. A melanoma cell line has been developed whose growth appears to be markedly enhanced by androgens. Rat ovarian cell lines have been developed whose growth and viability are hormone dependent in vivo and in vitro.

[Cancer Chemother Rep 59:47–49, 1975]

RESULTS AND DISCUSSION

With the goal of obtaining minimal-deviation cell culture lines from human prostatic tumors, we have established a colony of nude (athymic) mice in our laboratory. The colony is maintained under specific pathogen-free conditions and animals remain healthy for extended periods (at least 8 months, the age of the mice first born in the colony). We have had no problems with the "wasting" phenomenon observed in nude mouse colonies less stringently maintained.

Thus far, eight separate specimens of human prostatic tissue have been studied, including benign hyperplastic tissue and carcinomas, both local and metastatic. In all cases, tissue was placed in culture and epithelial outgrowth into monolayers was observed. Cells from these monolayers have been injected into the nude mice, both subcutaneously (sc) and in the spleen. The recipient mice have all been males bearing a testosterone implant.

Preliminary attempts to grow tissue from sc injected single-cell suspensions have been unsuccessful. Neither tumors nor the original cells could be recovered after periods ranging from 3 to 7 weeks. We have investigated the growth of a number of other tumors in nude mice and have found that the spleen is a superior site for implantation. Cells can be recovered at early stages after injection, and tumor growth is observed in the spleen when the same cells do not grow sc. We now have a number of animals in which prostatic cells have been injected in the spleen and allowed to grow for up to 3 months. Other approaches we have initiated to obtain growth of prostatic tissue in nude mice are: (*a*) implantation of cultured pieces of tissue rather than the monolayer of cells, (*b*) the use of neonatal nude mice (in which we have indications that better growth of implants occurs) as recipients, and (*c*) direct implantation of primary tissues.

In the expectation that human tumor cells will grow in nude mouse spleens, we have

[1] Department of Biology, University of California at San Diego, La Jolla.

[2] *Reprint requests to*: Dr. Gordon Sato, Department of Biology, University of California at San Diego, La Jolla, Calif 92037.

developed procedures to reisolate these cells after growth in the animal. We have prepared antisera to mouse cells in rabbits and absorbed the sera with human cells. Such antiserum is selectively cytotoxic, killing more than 95% of mouse cells while permitting survival of at least 60% of human cells in a parallel experiment. We have recently used such sera to treat spleen cultures from animals injected with human prostatic tissue. A small number of cells from the spleen culture survived. These putative human cells were then reinjected into nude mice. In the future, some surviving cells will be maintained in culture and characterized as to karyotype and hormone dependence.

A possible model system for androgen-dependent tumors has been discovered with the development, in this laboratory, of two clonal strains of mouse melanoma cells in culture. The first strain metastasized extensively in over 50% of the host animals while the second strain metastasized only rarely and less extensively (only a few discrete metastatic nodules). The nonmetastatic line seems to grow much faster in males than in females, requires androgens for growth in culture, and has androgen receptors. When this tumor is injected intravenously it seems to have some specificity in its site of growth. In six of ten animals, the largest tumor nodules were found in the testes (fig 1). We believe that the rapid growth in the testes reflects growth enhancement mediated by androgens. The metastatic line does not contain androgen receptors and seems to be independent of androgens. The metastatic line is also different in that its content of a particular globoside is less than one tenth that of the nonmetastatic line.

The availability of nude mice has also enabled us to begin considering the important

FIGURE 1.—Tumor mass growing in testis of mouse injected intravenously 3 weeks previously with B16 melanoma-derived cell line.

changes associated with transformation, and to possibly develop an immunologic approach to cancer therapy. Cytochalasin B-resistant SV40-transformed mouse cells appear to be more normal than parent transformants in that they are resistant to cytochalasin B, and have some or all of the following properties of nontransformed cells: growth to lower saturation density, higher serum requirement, lack of T antigen, and partial loss of SV40 DNA sequences (1). When they are injected into normal syngeneic hosts they seem to be normal, since they do not grow in the animals in which

TABLE 1.—Growth of transformed fibroblasts and cytochalasin B-selected revertants in animals

Host animal	No. of tumors/No. of animals injected with—				
	SVT2 (transformed)	Cytochalasin B revertants			3T3 cells (normal)
		CB1	CB2	CB3	
BALB/c mice	10/10	0/8	0/8	2/8	0/8
Nude mice	10/10	10/10	10/10	10/10	0/10

transformed cells grow. However, when cells are injected into nude mice, a surprising result is obtained: both transformants and cytochalasin B-selected cells form tumors although normal fibroblasts still do not grow (table 1).

This result means that the cytochalasin B-resistant cells have not regained normal growth control and that the "revertant" characteristics acquired with cytochalasin B selection are not related to the loss of the original transformed state. It also suggests that drug-resistant cells have enhanced immunogenicity, a conclusion supported by further experiments. If normal animals previously injected with cytochalasin B-resistant cells are challenged with transformed cells, they fail to give rise to tumors.[3] A distinct possibility, which requires further investigation, is that cytochalasin B-resistant mutants of tumor cells can be developed from any tumor and can be used to immunize the host against his tumor.

We have recently developed rat ovarian cells which require hormones for growth both in vitro and in vivo (2).[4] We have isolated, from luteinizing hormone, a factor which is active at concentrations of 10 ng/ml. These observations are not trivial. In fact, it is unprecedented to have a cell line which requires hormonal conditioning of the host to grow in vivo. We believe that this is an important necessary step in understanding physiologic growth regulation. Of immediate interest to our present work is that these cells die rapidly in hormone-depleted medium (fig 2). "Normal" 3T3 cells do not die in serum-depleted medium but remain quiescent and resume growth when serum is restored to the medium. We believe that this behavior is a physiologic analog of the morphologic phenomenon observed with polynuclear cell formation by cancer cells in the presence of cytochalasin B. In this case, in cancer cells

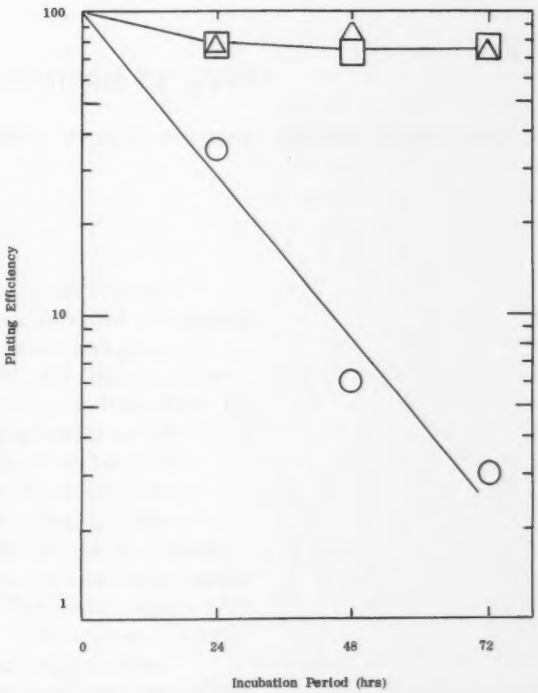

FIGURE 2.—Effect on plating efficiency of a prolonged period of incubation in depleted medium. Ovarian cell lines were plated in various media, then shifted to rich medium after indicated periods. ○ = Depleted medium alone; △ = depleted medium with 88 μg/ml serum growth factor; □ = depleted medium with 5 μg/ml partially purified luteinizing hormone (NIH-LH9).

hormone depletion blocks certain cell processes while others continue in an uncoordinated manner. This unbalanced situation culminates in cell death. It therefore seems likely to us that a study of specific growth-requirement factors for cancer cells is an important approach to cancer therapy.

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Properties of Prostatic Cultures Transformed by SV40 [1]

David F. Paulson,[2,3] Robert A. Bonar,[2] Yousuf Sharief,[2] Juan R. Vergara,[2] Charles Reich,[2] and Keerti V. Shah [4,5]

SUMMARY

SV40-transformed hamster prostatic tissue has been previously evaluated as a model for human prostatic carcinoma. Because the original cell line was lost, Syrian golden hamster prostatic tissue has been established in explant culture and infected with a 10^6-cell tissue culture infectious dose (50% effective) of SV40. After in vitro transformation, the cells were produced in quantity and 60×10^6 cells were injected into adult male Syrian golden hamsters 24 hours after 400 rads of whole-body radiation. After 60–90 days, a small palpable tumor developed. These tumors could be serially transplanted in adult male animals without immunosuppression. The tumor cells were established in tissue culture and the cells were returned to adult animals without immunosuppression where they rapidly produced fast-growing tumors. The solid tumors were composed of sheets of pleomorphic polygonal cells with large nuclei and many nucleoli; they resembled undifferentiated human prostatic carcinoma. In vitro, the cultures contained small, rapidly growing cells with a population doubling time of about 1.3 days. The cells carried the SV40-specific antigen. The modal chromosome number was 66–68 with a distribution of 47–120. Cells exposed to 2-bromo-5′-deoxyuridine in culture did not release particles with RNA-dependent DNA polymerase activity. Endocrine sensitivity in vivo and in vitro is undetermined to date.

[Cancer Chemother Rep 59:51–55, 1975]

In vitro transformation of mammalian prostatic tissues by SV40 was reported initially by Paulson, Rabson, and Fraley (1). Tissues from the ventral, lateral, and dorsal prostatic lobes of neonatal Syrian golden hamsters were established in tissue culture and exposed to SV40. After transformation, the cells were returned to subcutaneous sites in adult male hamsters where they produced soft hemorrhagic tumors which grew rapidly, metastasized to the periaortic lymph nodes and lungs, and killed 100% of the animals (1,2). The tumors were composed of sheets of pleomorphic polygonal cells with very little fibrous stroma. There was neither epithelial differentiation nor gland formation. Tumor-bearing animals had elevated levels of serum prostatic acid phosphatase which returned to normal after estrogen administration. Diethylstilbestrol [6] produced cytoplasmic vacuolization of the tumor cells and retarded tumor

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[2] Division of Urology, Department of Surgery, Duke University Medical Center, Durham, NC.

[3] Veterans' Administration Hospital, Durham, NC.

[4] Department of Pathobiology, The Johns Hopkins University, Baltimore, Md.

[5] *Reprint requests to*: David F. Paulson, MD, Division of Urology, Department of Surgery, Duke University Medical Center, Durham, NC 27710.

[6] Diethylstilbestrol: NSC-3070; 4,4′-stilbenediol, α,α′-diethyl-.

growth (3). Recently, Abdalla and Oliver (4) confirmed the endocrine sensitivity of this tumor model. With continued passage, the transformed prostate cells became progressively less responsive to hormonal control and it was felt desirable to attempt to establish again a new cell line. The subsequent isolation of viruses related to the oncogenic papovavirus SV40 from human brain and urine and the proposition that one of these, the BK virus, might exist as a latent urinary tract infection prompted the studies described herein (1,5,6). Not only was an endocrine-responsive cell sought, but the resultant cell was to be well characterized so that it might be used later in immunologic test systems.

METHODS AND RESULTS

Neonatal male Syrian golden hamsters were killed within 1 week of birth, and the prostatic tissues were harvested and established as explant cultures using medium 199 with 20% heat-inactivated fetal calf serum. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. When the cells began to migrate from the explants, one half of the tissues were infected with a 10^6-cell tissue culture infectious dose (50% effective) of SV40, the remaining dishes being maintained as virus-free controls. The virus-free controls failed to become confluent and did not survive their first trypsinization. Evidence of transformation was detected in the infected cultures within 2 weeks and was identified by accelerated growth and loss of contact inhibition. The cells were small and epithelial in character, with a population doubling time of 1.3 days (figs 1 and 2).

Two adult male hamsters received 400 rads of whole-body radiation and 24 hours later were injected subcutaneously (sc) with 60×10^6 cells. One animal developed a palpable tumor. This tumor was soft and vascular and was composed of small, epithelial-like cells with pseudo-gland formation (fig 3). The tumor subsequently has been passed by serial sc implantation in nonradiated male hamsters and has been maintained in vivo. Fragments of the tumor were trypsinized and established in tissue culture. Alternating in vivo and in vitro passage can be accomplished without difficulty.

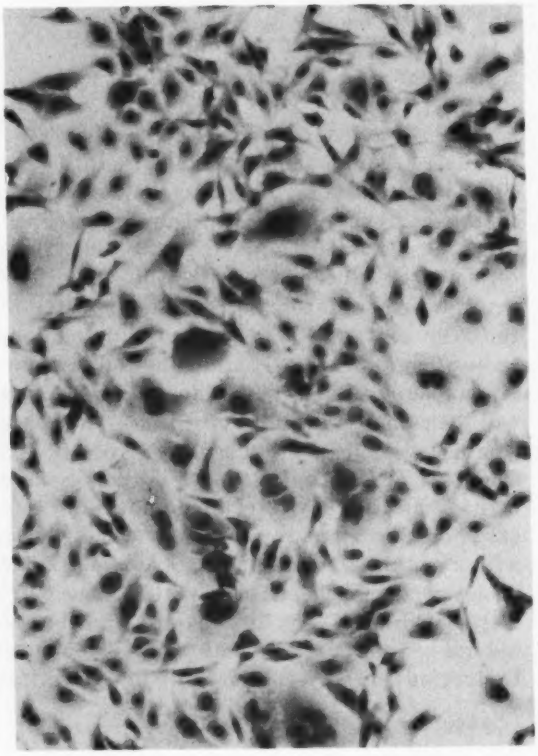

FIGURE 1.—Photomicrograph of SV40-transformed hamster prostate tissue in vitro (× 240).

Cells in culture were treated with colchicine [7] at a final concentration of 0.5 μg/ml for 3 hours. After harvesting with trypsin, the cells were treated with hypotonic KCl, fixed, air-dried, and stained with Giemsa according to Hungerford (7).

[7] Colchicine: NSC-757; CAS reg. No. 7006-15-7.

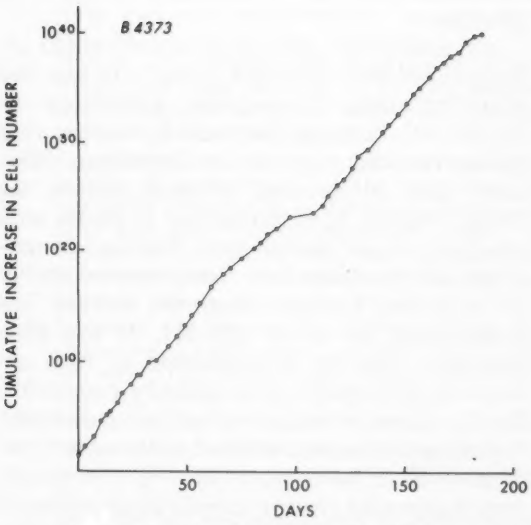

FIGURE 2.—Cumulative growth curve.

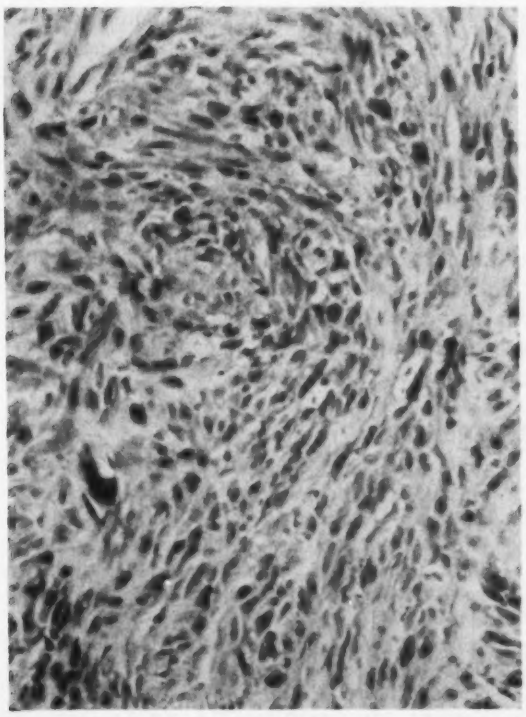

FIGURE 3.—Photomicrograph of tumor arising after sc injection of SV40-transformed hamster prostate cells (× 240).

The modal number of chromosomes (66–68) was near triploid, and the range was 47–120. The distribution of chromosome numbers in 100 mitoses is shown in figure 4. Many chromosome abnormalities were noted, including breaks and deletions with dicentric and ring chromosomes. Endomitosis and chromosome pulverization were seen occasionally. Paired fragments which varied in number and size were seen in all metaphase figures that had pulverized chromosomes and were found also in some figures without chromosome pulverization. Such paired fragments have been described as microchromosomes (8) and as double-minute chromosomes (9).

Fluids taken from cells in culture were tested for the presence of particles possessing RNA-directed DNA polymerase (reverse transcriptase) activity which is characteristic of the RNA tumor viruses. Medium removed from the cultures was centrifuged for 10 minutes at 10,000 rpm to remove whole cells and large debris. Six ml was then layered over a cushion of 20% glycerol and centrifuged for 35 minutes at 37,000 rpm, conditions sufficient to sediment known type-C RNA tumor viruses. The pellet was treated with the detergent Nonidet NP-40 (a gift of Shell Chemical Co.) and assayed for polymerase activity, with the hybrid templates poly(rA) · oligo$(dT)_{10}$ and poly(dA) · oligo $(dT)_{10}$ being used to provide greater sensitivity (10, 11). No activity was found in fluids from the routine cultures. Some cultures were treated for 2 days with 1×10^{4} M 2-bromo-5′-deoxyuridine (BrdU), a procedure known to stimulate virus particle production in a variety of cells carrying endogenous type-C RNA tumor virus

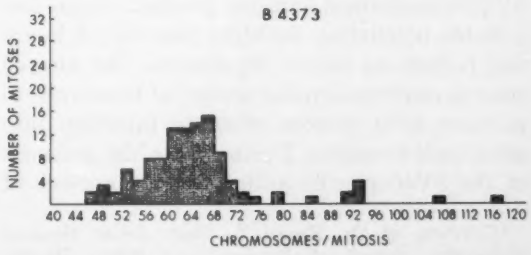

FIGURE 4.—Distribution of chromosomes in 100 mitoses.

(12). No evidence was found for release of enzyme-containing particles in the 1–10-day observation period after BrdU treatment.

The cells were grown on coverslips, acetone-fixed, air-dried, and examined for the presence of the SV40 tumor antigen (13).[a] For morphologic study, tissue culture cells grown on coverslips were fixed with 5% gluteraldehyde in cacodylate buffer and stained with May-Grünwald-Giemsa.

DISCUSSION

When most untransformed cells are plated in vitro, they will grow to a certain density and then either stop dividing or divide at a greatly reduced rate. Under identical conditions, the corresponding transformed cell will continue to multiply and may reach saturation densities ten to 25 times greater before being finally limited by nutritional factors. The culture characteristics of SV40-transformed hamster prostatic tissue were anticipated. After infection and alteration, the cells exhibited morphologic alteration, accelerated growth, and a lack of contact inhibition. In the unaltered state, cell-with-cell contact plays a role in inhibiting cell division, the individual cells in in vitro culture responding to the proximity of other cells by ceasing to multiply (14). This has been termed "contact inhibition." It has since been shown that unidentified serum factors play a role in controlling cell multiplication in tissue culture and that the addition of fresh serum to a nondividing culture of nontransformed cells will initiate division with loss of contact inhibition (10,15,16). Viral transformation reduces a cell's serum dependence, and those cells which are stably transformed have the ability to multiply continuously in factor-free serum. This line of SV40-transformed hamster prostatic tissue has a stable population doubling time of 1.3 hours and reflects no serum dependence. The altered state is confirmed by the ability of these cells to produce solid tumors after sc injection into adult male hamsters. Furthermore, the presence of the SV40-specific antigen in the nucleus of these cells confirms the presence of the SV40 genome.

As anticipated, significant chromosomal abnormalities were observed. After infection and transformation, chromosomal alterations occurred both in morphology and in number, thus giving rise to a very diverse karyotype. However, after 40 passages in tissue culture, the modal number of chromosomes is 66–68 with relatively broad distribution. Similar chromosomal abnormalities have been reported earlier in a Syrian hamster renal cell culture line transformed by SV40 (17–20). It has been suggested that the incorporation of viral genome in the hamster cells might be responsible for the chromosomal and karyotypic instability.

The recent demonstration of endogenous type-C particles in many cell types urged investigators to carry out reverse transcriptase assays to determine if endogenous hamster type-C viruses could be identified. The failure to demonstrate enzyme activity after BrdU stimulation would argue for the absence of endogenous type-C viral infection within these cultured cells.

Our previous studies with the SV40-transformed hamster prostatic tissue indicated that this might be an adequate modal system. The loss of endocrine sensitivity with prolonged in vivo passage coupled with the difficulties inherent in the intact animal prompted this attempt to duplicate the in vitro transformation. The subsequent cell line has been carefully characterized in hopes that the system can be used in vitro to unravel the complexities of the tumor in vivo.

[a] Courtesy of Dr. Keerti V. Shah, Johns Hopkins University, School of Hygiene and Public Health, Baltimore, Md.

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Etiology and Prevention of Prostatic Cancer

George B. Hutchison [1,2]

The principal remarks that can be made about the epidemiology of prostatic cancer are negative ones. There are very few ways to predict for most of you whether you are more or less likely than your colleagues to acquire this disease. Even less can be said about what you should do to alter this risk. This contrasts with coronary heart disease, for which the American Heart Association can give each of us an individual risk estimate (1). Probably no two people in this room will have the same estimate. Furthermore, it is suspected that we can alter our true risk by altering the factors involved in this risk estimate, principally diet, but also blood pressure, exercise, smoking, and other factors.

With respect to carcinoma of the prostate, it is clear that all men have a very considerable risk of this disease (a lifetime risk of about 2%), but very little can be said as to what groups of men have either distinctly higher or lower risks (2). Essentially no advice can be given as to how to avoid this disease. The positive facts that are known seem to be the following. The disease incidence increases with age through the most advanced ages for which data are available. It is more common in blacks than in whites in the United States, and the incidence rates for blacks may be the highest of any sizable population in the world (3). There is a great deal of international variation, with Japanese men having one of the lowest rates (4). Dr. Jackson and his colleagues have noted an increased risk in men with early puberty and coitus (5).

All other associations are either inadequately supported or are negative. Some of these, however, are sufficiently suggestive, reasonable, and important to deserve further investigation. Widowed and divorced persons have higher rates than married or single persons (2), an observation also made for cervical cancer and generally interpreted as a measure of promiscuity. Fathers have a greater prostate mortality than do men of similar age who are not fathers (6). Persons with normal liver function have higher rates than those with cirrhosis (7), presumably reflecting an hormonal effect, with inadequate removal of estrogens.

Time-trend data for prostatic cancer indicate a rise between 1950 and 1968 in crude mortality in both whites and blacks in the United States, with a greater rise for blacks than for whites (8,9). The rise in mortality for whites is small and reflects only the increasing age of the population. Age-adjusted rates show a marked increase for blacks and a very small drop for whites (10). Age-adjusted incidence rates show a rise between 1947 and 1969 for both races (3). While the facts of these rises are clear, the interpretation is obscure. The problem is to distinguish the phenomenon from artifacts of data collection. Is there simply better ascertainment in recent years, or is some environmental factor producing a true rise in incidence or mortality or both? One argument is that the present greater rate for blacks as compared with the rate for whites is unlikely to be due to better case finding, since the adequacy of diagnosis in blacks is unlikely to be better than that in whites. Since the excess in reported mortality for blacks is only recent, it is argued that the trend over time must not be artifactual. An alternative explanation is that the excess rate for blacks over whites may be real and long standing, as opposed to a recent development, and the time trend may be an artifact. In an earlier decade, cancer-mortality reporting for blacks not only failed to indicate an excess, though an excess may actually have existed, but showed a deficit. Over the years, reporting has improved and the present data show approxi-

[1] Department of Epidemiology, School of Public Health, Harvard University, Boston, Mass.

[2] *Reprint requests to:* Dr. George B. Hutchison, Department of Epidemiology, School of Public Health, Harvard University, 677 Huntington Ave, Boston, Mass 02115.

mately the true comparison: a mortality rate greater for blacks than for whites. In partial support of this argument is the fact that the earlier incidence data (obtained in 1947) already showed an excess for blacks, at a time when mortality data showed a deficiency for blacks (3). The case fatality rate for blacks has always been lower than that for whites, which is consistent with mortality reporting poorer than incidence reporting.

Many factors that have been suspected as correlates of risk for prostatic cancer have shown no association when investigated. These include occupation (6), history of benign hypertrophy of the prostate (11), a large number of sophisticated anthropometric measures (6), socio-economic status, and a variety of other diseases (12). Clearly, further studies, some of which are presently in progress, may reveal associations with some variables that do not seem to be associated on the basis of the present study.

Dr. Jackson's report (5) agrees with many others with regard to the small number of positive findings. If it is accepted that mortality rates for blacks are higher than those for whites, it will be most important to know whether cases from the two groups are morphologically similar. The same question will be asked regarding the comparison of Japanese with American groups. If groups are morphologically similar, one may suppose a higher incidence represents a heavier exposure to some carcinogen common to both. If morphology is different, the hypothesis of different carcinogens seems more tenable.

It is even more important to obtain a clear understanding of the time trend. While the differences in rates according to race may be due to identifiable external exposures, they may equally well be genetically determined. A time trend within 20 years cannot be genetically determined. A major exposure change for blacks but not for whites in the United States over a period of 20 years should be identifiable and should become the object of a preventive program. This hope, of course, will be fruitless if, in fact, there has been no major change in true incidence in either race. The well-known phenomenon of occult carcinoma of the prostate (13) results in a situation in which an increased case-finding effort may result in a greatly increased observed number of cases in the absence of any true increase in etiologic exposures.

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Monolayer Cultures of Human Prostatic Cells[1]

M. Edward Kaighn and Merrill S. Babcock[2,3]

SUMMARY

The objective of this study was to isolate, characterize, and preserve normal prostatic epithelial and carcinoma cell lines. To date, four specimens have been acquired from patients with benign prostatic hypertrophy. Culture attempts have been successful, yielding a total of 32 cell lines. Three of these lines have been examined thus far and were found to have clearly demonstrable tartrate-inhibitable acid phosphatase granules by histochemical techniques. Several problem areas emerge from these preliminary studies. These involve the difficulty of (*a*) characterization of the cells as originating from prostatic epithelium, (*b*) identification of prostatic carcinoma cells, and (*c*) possible senescence in culture of cell lines.

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Most of the available information on the nature and behavior of prostatic tumors has been obtained from clinical observations and studies on animal models. Only limited success has been achieved in establishing cultures from prostatic tissues. Such cultures would be invaluable for studying candidate etiologic agents and for evaluating chemotherapeutic compounds.

Most attempts to culture normal or tumorous prostatic tissue have been disappointing, resulting, at best, in short-term maintenance. This is particularly true of epithelial cell types.

Since most tissues, normal or neoplastic, consist of a mixture of cell types, cells with a higher growth rate soon dominate the culture population. Thus, in even short-term cultures, fibroblasts rapidly replace epithelial cells.

In 1959, Rohl (1) reported growth of prostatic cancer cells but he was unable to obtain a uniform cell type. More recently, techniques which permitted maintenance of short-term cultures of prostatic tumor epithelium have been described. These efforts resulted in maintenance of cells from 9 days in organ culture (2) to as much as 7 months (3). In the latter case however, there was no indication of the morphology or other properties of the cells. Schroeder et al (4) were able to maintain prostatic epithelial cultures in monolayers for several months. Brehmer et al (5) reported that monolayer cultures prepared from trypsinized prostatic adenocarcinoma and benign hyperplasia could be subcultured ten to 12 times over a period of 3 months. Their cultures were morphologically heterogeneous.

Our approach in isolating homogeneous cell lines from prostatic tissue consists of plating enzymatically dispersed cell suspensions at low population densities so that single cells can form discrete colonies, thereby avoiding the problem of overgrowth by fibroblasts. These primary clones are then individually subcultured and grown into large populations. This approach has been successful in isolating

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[2] W. Alton Jones Cell Science Center, Lake Placid, NY.

[3] *Reprint requests to:* M. Edward Kaighn, PhD, W. Alton Jones Cell Science Center, Lake Placid, NY 12946.

specialized cell lines from a number of other sources (6,7).

A preliminary account of our application of primary cloning to the isolation of homogeneous cell lines from human benign hyperplasia will be given.

METHODS

Benign hyperplastic specimens were obtained from nearby cooperating hospitals. One normal prostate was obtained from Dr. Malinin of the Jackson Memorial Hospital, Miami, Florida. Immediately after surgery, tissue specimens were stored in complete medium at 4°C or on ice until cultured. The hyperplastic specimens were dissociated approximately 4–5 hours after removal from the patient, and the normal prostate was dissociated about 28 hours after removal. Portions of each specimen used to establish cultures were retained for acid phosphatase histochemical and conventional histologic studies. Primary cell suspensions were prepared as follows: The specimen was weighed and minced into 1–2-mm^3 pieces. The minced tissue was transferred to a 50-ml Erlenmyer flask and washed three times with Moscona saline containing 1% chicken serum. Approximately 10 ml of CTC (0.1% collagenase, 0.1% trypsin, and 1% chicken serum in Moscona saline) was added per gram of tissue. The flask was then incubated in a reciprocating water bath at 37°C with gentle agitation. After 20 minutes the tissue was allowed to settle. The supernatant, containing suspended cells, was transferred to a centrifuge tube, and an equal volume of chilled medium (F12K with 17% fetal calf serum) was added (8). This process was carried out five times. Optimal cell yields were generally obtained with the later extractions. The supernatant from each extraction was centrifuged at 800 rpm for 7 minutes and the resulting pellet was resuspended in fresh media.

Clonal cultures were established by inoculating 100-mm plastic petri dishes (Falcon) containing 6 ml of medium. The plates were fed three times a week with complete medium and incubated at 36.5°C with a 5% CO_2 atmosphere.

After 2 or 3 weeks, when they were 1–2 mm in diameter, single colonies were encircled with porcelain cylinders (Fischer Scientific) coated with silicone (Dow-Corning). The colony was suspended with CTC, and the cylinder contents were transferred to a 60-mm petri dish containing 4 ml of media. When nearly confluent, the cells were passed to 100-mm petri dishes and grown in sufficient number for freezing in liquid nitrogen (third or fourth passage). The cells were cultured in the presence of antibiotics (penicillin G, fungizone, and kanamycin) until each cell line had been frozen in liquid nitrogen. The antibiotics were then withdrawn.

ISOLATION OF CLONAL LINES

Using the above method, viable cell lines have been obtained in each of five attempts to date (table 1). Cell lines are either still in culture or have already been frozen. Cell morphology and growth rates vary from one cell line to the next (figs 1 and 2). The culture media

FIGURE 1.—Cell line HP1-7; phase-contrast photo of cells in culture.

FIGURE 2.—Cell line HP1-4; grown on a coverslip (hematoxylin and eosin stain).

FIGURE 3.—Cell line HP1-8; frozen on a coverslip and stained for acid phosphatase activity.

used, F12K with 17% fetal calf serum, has proven to be quite satisfactory. All lots of fetal calf serum used have been tested for toxicity and growth-promoting factors by a clonal method. In spite of our success with this media, we recognize a great need for work on the specific media requirements of human prostatic cells.

ACID PHOSPHATASE ACTIVITY OF CULTURED CELLS

We examined cell lines for acid phosphatase activity by a histochemical method as a means of tentatively identifying those lines derived from human prostatic specimens. Tartrate inhibition of acid phosphatase was studied since this was considered characteristic of the human prostatic enzyme (9,10). Some authors have expressed the opinion that the presence of abundant acid phosphatase alone is a suitable marker for prostatic cells in culture regardless of whether it is tartrate inhibitable or not (4). The procedure used in histochemical staining of monolayer cultures on coverslips is similar to that of Humason (11) with the following modifications: The substrate was naphthol-ASE-phosphate, fast blue RR salt was used, and the cells were counterstained with 0.5% eosin. To study tartrate inhibition, 0.01 M sodium tartrate was used in the incubation mixture. Since acid phosphatase is largely contained in the lysosomes (9), coverslip cultures were frozen to rupture the lysosomes just prior to acid phosphatase staining.

All prostatic cell lines examined for acid phosphatase had an abundance of granules indicating a high level of acid phosphatase activ-

FIGURE 4.—Cell line HP1-8; frozen on a coverslip and stained for acid phosphatase activity in the presence of 0.01 M tartrate.

ity (fig 3, table 2). When similar cultures were stained for acid phosphatase activity in the presence of 0.01 M tartrate, the inactivation was virtually complete (fig 4). Once it had been established that the prostate lines had abundant tartrate-inhibitable acid phosphatase, experiments with other cultured cell lines were carried out to determine its validity as a prostate-specific marker. A total of ten cell lines were tested. Three lines were of prostatic origin and the other seven were from various other sources (*see* table 2).

Five of the seven lines of nonprostatic origin had granules as abundant as those of the prostatic lines. In addition, in five of the seven nonprostatic lines, the acid phosphatase activity was virtually abolished by tartrate. Only one cell line, KBN, had abundant non-tartrate-inhibitable phosphatase. These results make it clear that acid phosphatase, whether or not it is tartrate inhibitable, does not constitute a suitable prostate-specific marker.

CONCLUSIONS

The objective of this project is to isolate, characterize, and preserve normal, hyperplastic, and carcinomatous prostatic cell lines. To date, four specimens later diagnosed as benign hyperplasia and one normal prostate have been acquired. Culture attempts have been successful, yielding a total of 32 cell lines. Problems most often associated with the establishment of prostatic monolayer cultures as useful model systems include (*a*) difficulties in culturing pure epithelial cell lines (3), (*b*) lack of appropriate criteria to identify cells originating from human prostatic epithelium, (*c*) choice of methods for characterizing the origin of human adenocarcinoma cell lines, and (*d*) definition of the nutritional requirements of human prostatic cells.

TABLE 1.—Cell lines derived from primary clonal culture

Specimen	Age of patient* (yrs)	Diagnosis†	Date of isolation	Cell lines isolated	Lines in liquid nitrogen to date	Lines currently in culture
HP1	57	BPH	3/28/73	17	17	0
HP2	52	BPH	4/11/74	3	0	3
HP3	66	BPH	4/16/74	4	0	4
HP4	70	BPH	4/16/74	8	2	8
HP5	NA	Normal	6/8/74	‡	0	0

*NA = not available.
†BPH = benign prostatic hyperplasia.
‡HP5 clones had not been picked at the time of table preparation.

TABLE 2.—Tartrate-inhibited acid phosphatase in selected cell lines

Cell line	Cell source	Incubation*	
		Normal	With tartrate
HP1-2	Human prostate, hyperplasia	+++	0
HP1-8	Human prostate, hyperplasia	+++	0
HP4-1	Human prostate, hyperplasia	++	0
CL9	Rat liver, normal	+++	0
LS	Mouse connective tissue	0	0
HeLa S-3	Human cervix, carcinoma	++	0
WI-38	Human lung, normal	+++	0
CF3	Human foreskin, normal	+++	0
KBN	Human oral cavity, carcinoma	+++	+++
L2	Rat lung, normal	+	0

*+ = ≤ 33% cells with acid phosphatase granules; ++ = 33%–66%; +++ = 66%–100%; 0 = no acid phosphatase granules.

The fact that the presence of acid phosphatase or its inhibition cannot be used as a specific marker for cultured cells of prostatic epithelial origin has been established. This leaves open the question as to what other methods may be useful. How cell lines of human prostatic origin (normal, hyperplastic, or carcinoma) respond to various hormones and antitumor agents will be studied. They will also be characterized with respect to clonal plating efficiency, growth rate, morphology, karyotype, and oncogenicity.

We are currently working towards the production of a prostatic acid phosphatase-specific antiserum, since recent work in this area has been quite promising (12). Other means of characterizing prostatic epithelial cells are also being considered, including their response to various hormones. Future work to involve prostatic adenocarcinomas will introduce yet another question as to how a cell can be specifically characterized as being of neoplastic origin.

Early studies with our prostatic cell lines that have now been in culture for 5 months or more reveal a marked decrease in the doubling rate of the cells accompanied by an increase in cell size. Whether this decrease in doubling rate will be a limiting factor in future prostatic lines and planned research has yet to be determined.

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Etiology and Prevention of Prostatic Cancer

Donald Merchant [1,2]

Since I am not prepared to review a specific topic as Dr. Hutchison has done and I do not have experimental results to present as Dr. Kaighn has done (see preceding papers), I will take this opportunity, as a member of the working cadre, to present some general remarks concerning the etiology and prevention of prostatic cancer.

When I was asked to become a member of the Prostatic Cancer Project at the outset of the Organ Site Program, I had reservations about such an approach to the study of cancer. I believe many others shared these feelings concerning the chances of success. While it is too early to be certain, the program presented at this workshop is strong evidence that my original assessment may have been incorrect, that my misgivings were not well founded, and that, indeed, we are coming to a point at which we can accomplish a great deal more by bringing diverse disciplines together to focus on the problem than we can by continuing to work on an individual basis.

From the things we have heard in this session, it is quite evident that we have been successful in drawing together a group of investigators with a great deal to offer in terms of perspective, basic knowledge, and technology. These investigators represent all the disciplines known to be related to prostatic cancer. Since we work in different disciplines and at different institutions, our paths do not necessarily cross in the normal course of events. Cross-fertilization of ideas and concepts related to prostatic cancer occurs on a limited and erratic basis. Moreover, we all recognize the difficulty of keeping abreast of the literature even in our own specific area of interest. I believe the contacts provided through this program already have begun to bear fruit to an extent that would not have been possible otherwise.

It is important, however, to point out that our task has only begun. We have succeeded in identifying the investigators, in establishing programs for all, and in bringing these individuals together. The important point, for the success of the program, is to stimulate participation in a truly cooperative effort on a continuing basis.

This program, as well as other organ site programs, can and should be far more significant as a whole than the sum of its parts. Cancer, of which prostatic carcinoma is a good example, represents a general problem in biology and clinical medicine. It is one that has many facets and any one individual working alone, or any laboratory focusing on one or two aspects, can easily miss the overall view, thereby losing sight of some of the important interactions we must understand and consider in order to move forward.

Our level of sophistication in technology and the extent of information in various fields now make it feasible for us to develop a program with (*a*) a free interchange of information, ideas, and materials, (*b*) good communication, and (*c*) frequent meetings allowing us to discuss results and problems. To be successful, however, we must be willing to go much further with an interchange than we have been accustomed to in the past. For example, those interested in viral oncology must not be content to keep up with just the investigators in that field, but in addition they must talk to colleagues working in endocrinology, chemical carcinogenesis, immunology, etc.

It is apparent to me that cancer is a multifaceted problem. With regard to the cause of cancer, I am convinced that we probably are not dealing with a single etiology or even perhaps a double etiology. We are dealing with a complex interaction, and unless we see it as that and freely exchange information and materials, we

[1] Department of Microbiology and Cell Biology, Eastern Virginia Medical School, Norfolk.

[2] *Reprint requests to:* Dr. Donald Merchant, Department of Microbiology and Cell Biology, Eastern Virginia Medical School, PO Box 1980, Norfolk, Va 23501.

will not make the progress that is possible.

I realize it will be extremely difficult for any one of us to keep in touch with all the groups involved, but this then becomes our (the working cadre's) responsibility. Our success ultimately depends upon your willingness to share information with others from the earliest time so that everyone knows what is going on and can profit from new information and advances in methodology. This also will make it possible to establish cooperative efforts between many of the projects. It will be very important to have several groups using the same source material to facilitate the understanding of interrelated factors. For example, information related to the endocrinologic, virologic, and immunologic status of the same samples might be put together to make a more meaningful picture. This approach could significantly shorten the time required to achieve answers to important questions.

Effect of 3-Methylcholanthrene (NSC-21970) on Human Prostate in Organ Culture [1,2]

Wilbur F. Noyes [3,4]

SUMMARY

Long-term organ culture of normal human prostatic tissue with demonstration of functional and morphologic maintenance of the glandular epithelium is described. The carcinogen 3-methylcholanthrene at a level of 4 μg/ml in the culture fluid induced morphologic changes that were not observed in untreated cultures. The changes observed after exposure to the carcinogen for 15–30 days included hyperplasia and anaplasia of the glandular epithelial cells with large variation in nuclear size and shape. These changes were suggestive of a neoplastic potential.

[Cancer Chemother Rep 59:67–71, 1975]

There have previously been a number of reports of the organ culture of human benign hyperplastic prostatic tissue with maintenance of good histology for periods of up to 9 days (1–4). The present report describes the maintenance of normal human prostatic tissue in organ culture for prolonged periods and the effect of the carcinogen 3-methylcholanthrene on the glandular epithelial cells of the cultured tissue.

MATERIALS AND METHODS

Normal prostatic tissue was obtained during surgery in cases of cystectomy and was immediately placed in Hank's balanced salt solution containing 10% fetal bovine serum at 4° C for transport to the laboratory. The patients' ages ranged from 50 to 70 years. The tissue was cut with a scalpel into pieces approximately 3 × 3 × 1 mm and two to four fragments were placed on pieces of gelfoam sponge (The Upjohn Co.) measuring 15 × 8 × 3 mm in a T-30 flask (Bellco). Five ml of media was added, consisting of McCoy's 5a plus 20% fetal bovine serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. The prostatic organ cultures were maintained as rocker cultures in an apparatus as described by McLimans et al (5). In the apparatus the T-30 flasks were held at an angle of 25 degrees from horizontal and rocked through 50 degrees every 3 minutes. The rocking motion provided a thin nutrient film and allowed adequate gas exchange. The gas phase was 95% O_2 and 5% CO_2. The culture medium was replaced and the cultures were gassed to maintain the pH level at approximately 7.2. For histologic examination, the cultured tissue was fixed in Bouin's fluid, serially sectioned at 5μ, and stained with hematoxylin and eosin.

3-Methylcholanthrene was dissolved in acetone and an amount was added to the culture media to give a concentration of 4 μg/ml. An equivalent amount of acetone was added to other cultures to serve as controls.

[1] Supported by Public Health Service grant CA–14387 from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

[2] 3-Methylcholanthrene: CAS reg. No. 56–49–5.

[3] Roswell Park Memorial Institute, Buffalo, NY.

[4] *Reprint requests to:* Wilbur F. Noyes, PhD, Department of Biological Resources, Roswell Park Memorial Institute, 666 Elm St, Buffalo, NY 14203.

RESULTS AND DISCUSSION

The prostatic tissue used in these experiments was histologically normal. Figures 1*a* and 1*b* are representative areas from two of the operative specimens which illustrate the normal histology of the tissue. Figure 1*c* illustrates the appearance of the prostatic tissue after 12 days in organ culture. Note the maintenance of the glandular epithelial cells and that the histology approaches that of the operative specimens. Glands at the periphery of the organ explant were maintained in good morphologic condition while those in the interior of the explant had frequently degenerated, probably due to a lack of penetration of nutrients and oxygen. Such a degenerative interior area can be noted in the central right area of figure 1*c*. The interior degeneration of the tissue could probably have been avoided by using smaller explants. However, the 3 × 3-mm size was preferred due to the greater probability of obtaining glandular elements since these were usually distributed irregularly in the operative specimens. Good epithelial cell morphology was maintained for extended periods in culture. Figure 1*d* illustrates human prostatic tissue that was maintained for 43 days in organ culture. Here, some flattening of the epithelial cells is evident and there is also a marked loss of cells from the connective tissue. In some areas of the long-term cultures, squamous metaplasia was evident in some of the glandular elements.

The organ-cultured prostatic tissue maintained some degree of functional activity and this was demonstrated by the secretion of neutral mucin, as determined by PAS staining (6), by the glandular epithelial cells of the cultured prostate. Figure 1*e* illustrates a 12-day culture stained by the PAS method and lightly counterstained with hematoxylin. Neutral mucin appears as darkly stained material in the apical portion of the epithelial cells and in the lumen of the gland. Acid phosphatase production, another measure of function, was demonstrated using Gomori's method (7). Figure 1*f* illustrates production of this enzyme by the glandular cells of prostatic tissue maintained for 12 days. Note the black deposits of lead sulfide in the epithelial cells.

The effect of 3-methylcholanthrene on cultures derived from three different normal human prostatic specimens was examined. An initial effect of 3-methylcholanthrene on the organ cultures was noted at 8 days, when a sparcity of epithelial cells lining the acini was observed in some areas of the cultured tissue.

After 15–30 days of exposure to 4 μg/ml of 3-methylcholanthrene, examination of sections of the explants revealed changes in the morphology of the glandular epithelium as compared with untreated cultures. The areas of altered cells were irregularly distributed throughout the cultured tissue and examination of serial sections was necessary for proper evaluation. The changes occurred in approximately 5%–10% of the glandular acini and varied from groups of epithelial cells with increased intensity of staining and moderate hyperplasia, to those with marked hyperplasia. The changes also included anaplasia with a large variation in nuclear size and shape. Figures 2*a* and 2*b* illustrate a group of acini showing squamous metaplasia in an organ culture treated for 30 days with 3-methylcholanthrene. In some areas of the acini the cells show increased intensity of staining and marked variation in nuclear size and shape; one of these areas is enlarged in figure 2*b*. Figures 2*c* and 2*d* are from an organ culture exposed to 3-methylcholanthrene for 15 days. The area illustrated shows hyperplasia of the glandular epithelial cells. Some variation in the nuclear size and shape can be detected in the higher magnification, as shown in figure 2*d*. Figures 2*e* and 2*f*, from a culture treated with 3-methylcholanthrene for 15 days, demonstrate an acinus with anaplastic changes in some of the glandular cells. Note the loss of differentiation, the extreme variation in size and shape of the nuclei, and also the presence of very large nuclei.

The morphologic changes in the glandular epithelial cells induced by exposure to 3-methylcholanthrene, particularly as illustrated by figures 2*e* and 2*f*, are suggestive of a possible neoplastic potential. Studies to assess the neoplastic potential of carcinogen-treated organ cultures in immunosuppressed animals are under investigation.

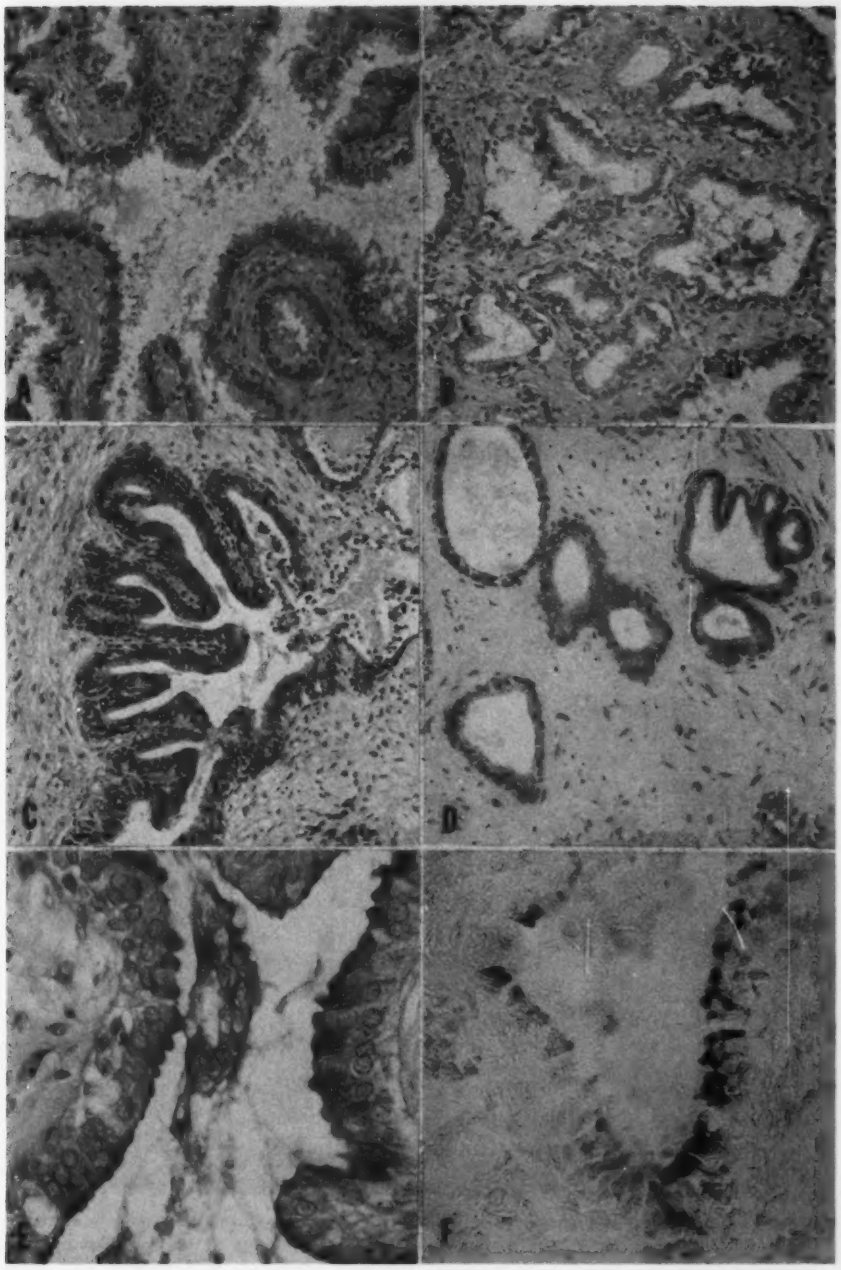

FIGURE 1.—(*a* and *b*) Normal human prostatic tissue as received from surgery (hematoxylin and eosin stain, × 100). (*c*) 12-Day organ culture of human prostate. Note maintenance of glandular epithelium (hematoxylin and eosin stain, × 100). (*d*) 43-Day organ culture of human prostate. The glandular epithelium is maintained but somewhat flattened and there is extensive loss of cellularity from the stroma (hematoxylin and eosin stain, × 100). (*e*) 12-Day organ culture, PAS stain for neutral mucin. The mucin is darkly stained material in the apical portion of the cells (× 250). (*f*) 12-Day organ culture. Frozen section stained for acid phosphatase by Gomori's method. Note deposition of lead sulfide in epithelial cells (× 250).

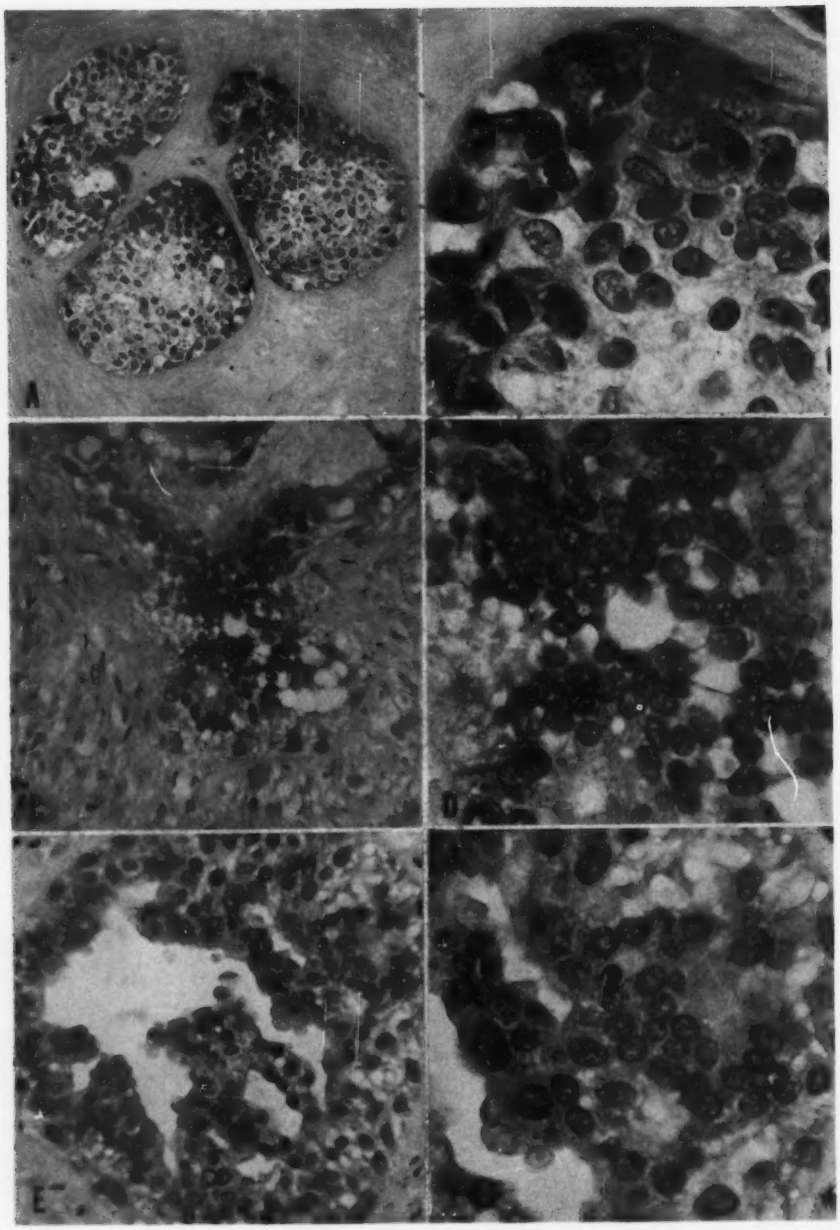

FIGURE 2.—(*a*) 3-Methylcholanthrene-treated organ culture (30-day exposure) showing group of acini with squamous metaplasia. Some of the cells have marked variation in nuclear size and shape and increased staining intensity (hematoxylin and eosin stain, × 100). (*b*) Same as fig 2*a*. Higher magnification of cells showing variation in nuclear size and shape (hematoxylin and eosin stain, × 640). (*c*) 3-Methylcholanthrene-treated organ culture (15-day exposure) showing hyperplastic area of glandular epithelium (hematoxylin and eosin stain, × 250). (*d*) Same as fig 2*c*, higher magnification (hematoxylin and eosin stain, × 640). (*e*) 3-Methylcholanthrene-treated organ culture (15-day exposure) illustrating an acinus with marked anaplastic changes of glandular epithelium. Note extreme variation in nuclear size and shape (hematoxylin and eosin stain, × 250). (*f*) Same as fig 2*e*, higher magnification (hematoxylin and eosin stain, × 640).

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General Discussion: Etiology and Prevention of Prostatic Cancer [1,2]

W. Winkelstein: I would like to make a few comments concerning the remarks of Drs. Jackson and Hutchison (1,2). I think it's very clear that the most striking epidemiologic characteristic of prostatic cancer is the difference in incidence between blacks and whites in this country. In the course of the investigation of the association between ambient air pollution and mortality in Buffalo, New York, my colleagues and I noted that in the white population under the age of 70 there was a strong inverse association between economic status and prostatic cancer; that is, the mortality was much higher in the lower economic groups. This led us to hypothesize that the black-white difference might simply be a reflection of the generally low economic status of blacks in our society and we began investigating that issue first in the Buffalo population. Unfortunately, there was not sufficient population for us to test the hypothesis, and we have been looking at it now in the Alameda County population. Unfortunately again, I don't have the results at this time. It seems to me that while both Drs. Jackson and Hutchison indicated that occupational associations have not been established, some of the work from Great Britain would suggest that we should continue to investigate this possibility. Also, the fact that in the older population the economic gradient I mentioned is reversed would suggest several things, one of which is that in younger groups there may be a different disease or that the causes might be different in different ages. Dr. Merchant mentioned (3) that there are many possible causes of this disease, and I would also like to say again that the area of chemical carcinogenesis certainly deserves exploration in view of the economic differences and the observation in Great Britain of certain occupational associations. I wouldn't want to give it very much emphasis, but in the two major studies of air pollution and disease that have been conducted in the United States (one in Nashville, Tennessee, and one in Buffalo, New York), a substantial association between levels of particulate pollution and distribution of prostatic cancer in the community was demonstrated. This association was not universal for all cancers but was the same in both communities for stomach cancer and prostatic cancer. Again, this association suggests the need to look into the possibility of chemical carcinogenesis.

D. Byar: The association between air pollution and cancer of the prostate has always fascinated me, but it sounds like a potentially classic case of an effect mediated through an as yet unidentified variable. From the deepest alveolus of the lung to the prostate is a long way for any sort of direct action, but if being at a high risk from air pollution means being at a high risk for something else, it might certainly be worth pursuing.

W. DeWys: In Dr. Jackson's data (1) one notes a high frequency of histologic grade I cases. I would question whether this material has been submitted to other pathologists for confirmation of the distinction between grade I

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[1] The following investigators participated in this discussion: *W. Winkelstein* (University of California at Berkeley), *D. Byar* (National Cancer Institute, Bethesda, Md), *W. DeWys* (Northwestern Medical Center, Chicago, Ill), *S. Shain* (Southwest Foundation for Research and Education, San Antonio, Tex), *F. Rapp* (The Pennsylvania State University, Hershey), *H. Rosenthal* (Roswell Park Memorial Institute, Buffalo, NY), *M. Jackson* (Howard University College of Medicine, Washington, DC), *M. Heshmat* (Howard University College of Medicine, Washington, DC), *Z. Hruban* (University of Chicago, Ill), *M. Webber* (University of Colorado Medical Center, Denver), *M. Pollard* (University of Notre Dame, Ind), *R. Gittes* (University of California at San Diego School of Medicine), *M. Tannenbaum* (Columbia University, New York, NY), *W. Voight* (University of Miami School of Medicine, Fla), *W. Panko* (University of Virginia School of Medicine, Charlottesville), *M. Kaighn* (W. Alton Jones Cell Science Center, Lake Placid, NY), *J. Horoszewicz* (Roswell Park Memorial Institute, Buffalo, NY), *G. Sato* (University of California at San Diego, La Jolla), *P. Siiteri* (University of California at San Francisco School of Medicine), *D. Lubaroff* (University of Iowa Hospitals and Clinics, Iowa City), *L. Dmochowski* (M. D. Anderson Hospital and Tumor Institute, Houston, Tex), and *K. Maruyama* (M. D. Anderson Hospital and Tumor Institute, Houston, Tex).

[2] *Reprint requests to:* Mrs. Pamela W. Hutchins, *Cancer Chemotherapy Reports*, National Cancer Institute, Blair Bldg, Rm 3A05, Silver Spring, Md 20910.

neoplasia and atypical or hyperplastic tissue. If, for example, in the black population being studied there are grade I cases that might be called non-neoplastic by other pathologists, this would increase the reported incidence of prostatic cancer in blacks. Inclusion of such cases would have little effect on population-based mortality rates. Inclusion of such cases could then underlie the reported increased incidence of prostatic cancer in blacks compared with whites while the mortality rates show little difference.

S. Shain: I was wondering, Dr. Jackson, about the method you used for determining plasma testosterone levels. Was the determination performed with whole plasma extract only? The reason I raise this point is that last year the Endocrine Group at McGill University demonstrated an apparent age-related change not in the total plasma testosterone concentration but in the distribution of testosterone between bound and free-form, and therefore a change in the amount of active testosterone available in the plasma. Their data correlated very nicely in that those individuals showing increased levels of bound plasma testosterone (ie, less free plasma testosterone) evidenced increased plasma luteinizing hormone levels so that there would seem to be a correlation between biologic activity and bound and free testosterone. One might wish to investigate a possible correlation between your cases of prostatic cancer and the bound and free levels of plasma testosterone.

F. Rapp: Many at this conference feel that herpes simplex viruses may play some role in prostatic carcinoma. Since herpes simplex virus type II is venereally transmitted, I wonder whether there is a correlation between the venereal disease incidence in these various populations and cancer of the prostate. More importantly, what is the venereal incidence in the Nigerian population examined versus that of the Washington, DC, population?

H. Rosenthal: I would like to address myself to the remarks of Dr. Hutchison (2). It occurred to me when I listened to his exposé concerning the relationships between the sex life of the male and prostatic cancer, that all our American blacks are of mixed race. Have there been any other statistics on the incidence of prostatic cancer in mixed races, for example, from Brazil?

M. Jackson: Dr. DeWys questioned the morphologic criteria used for separating well-differentiated carcinoma from non-neoplastic proliferative lesions of the prostate. For the diagnosis of the usual adenocarcinoma, we use established morphologic criteria, namely, disturbance of normal architectural patterns by small acini lined by a single layer of cells with slight nuclear variation and arranged in a back-to-back pattern with loss of the periacinar fibromuscular band. Problem cases are evaluated by at least three pathologists, and if the problem is not resolved we frequently obtain outside consultation. However, borderline cases are usually underdiagnosed and the clinician is advised to follow the patient closely. Only those patients with established diagnosis of carcinoma are entered into the study. Therefore, I am confident that the increased incidence of carcinoma in our material is real and is not a reflection of overdiagnosis. I would be delighted to have other pathologists, experienced in this area, review our material and offer their comments. Dr. Shain, I cannot give you the method used for testosterone determination. Our specimens are analyzed at the Worcester Foundation for Experimental Biology and it is my understanding that radioimmunoassay is the basis of their method. The total plasma testosterone concentration is measured and not the bound and free fractions. The third question related to herpes simplex virus and the relationship of venereal disease to carcinoma of the prostate in the populations studied. Dr. Heshmat, who is the epidemiologist in our group, has recently completed some studies comparing venereal disease and carcinoma of the prostate in Scandinavians. I will ask Dr. Heshmat to comment on this last question.

M. Heshmat: As for the relationship or association between venereal disease and prostatic cancer in our study, we found that the incidence of venereal disease in the 21 matched pairs was high. We had 13 patients who had a history of venereal disease as compared to 12 controls. Therefore, we cannot say there is

any association. However, I have seen the figures for the incidence of gonorrhea in Denmark for the period 1890–1970 and also the mortality figures from cancer of the prostate for 1940–1970. From these figures I drew trend curves and superimposed them. The curves matched very well with a lag period of about 40 years. This, of course, could be an artifact, but there may be a real association between venereal disease and cancer of the prostate gland.

D. Byar: I would like to recommend Dr. Heshmat's publication (4) with Dr. Jay Herson on the surprising coincidence of these gonorrhea incidence curves and the incidence curves for prostatic cancer with a lag period of about 40 years. I distributed copies of this article to the members of the working cadre because, unfortunately, it was published in the Annals of the D. C. Medical Society, a journal with limited circulation. I am sure Dr. Heshmat would be glad to supply copies upon request. We will now proceed to a discussion of the other topics, and I'll first recognize Dr. Hruban.

Z. Hruban: Several years ago the presence of intracisternal tubular structures was reported in various tissue cells from patients with arthritis and collagen disease and in certain neoplastic cells. These cells were believed to be of viral origin. It is of interest that these structures are commonly found in the prostates of dogs, particularly in vascular endothelium and macrophages (fig 1). Less frequently they are seen in smooth muscle cells, autonomic nerve fibers, and glandular epithelium. In addition to tubular reticular structures we observed intracisternal rod-shaped structures (fig 2). One of the prostates we studied contained distemper virus (fig 3). In other studies we observed the intracisternal tubular structures in kidneys of *Ateles geoffroyi* and in liver cells of *Cebus albifrons*. I would like to ask Dr. Dmochowski whether these tubular structures are similar to those he observed in human prostates and how he interprets them. I do not believe that they are viruses and there is recent evidence that they are related to immunoglobulin synthesis (5).

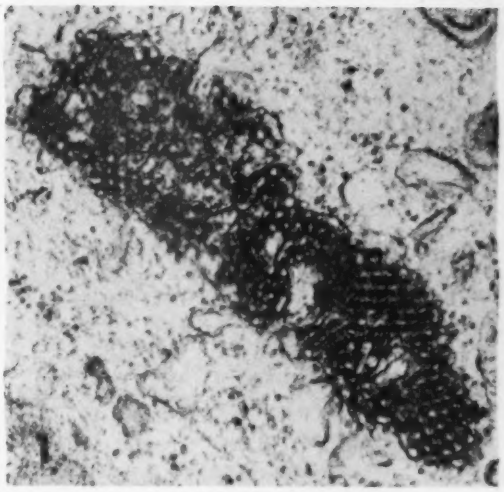

FIGURE 1.—Intracisternal tubular structures found in the prostates of dogs.

M. Webber: I would like to continue the discussion by Dr. Rapp concerning herpesviruses and cancer. I will present my observations on possible virus-associated cytopathic effects (CPE) in human prostatic epithelium grown in vitro. First, I would like to demonstrate how we culture prostatic epithelial cells. The tissue is derived from normal benign or malignant prostates. Normal tissue is obtained from organ transplant donors. The tissue is collected and cut into 0.5–1 mm^3 explants. Prostatic tissue before culture is shown in figure 4*a*. Epithelium

FIGURE 2.—Intracisternal rod-shaped structures found in the prostates of dogs.

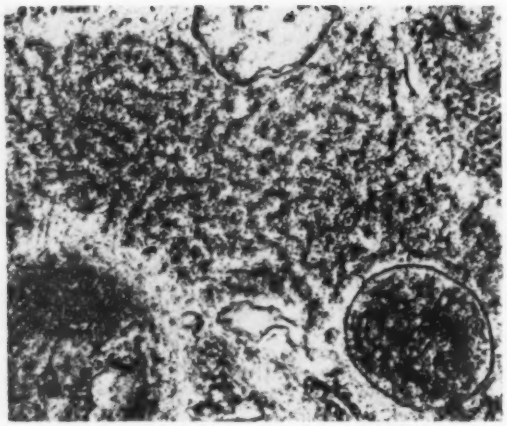

FIGURE 3.—Distemper virus contained in the prostates of dogs.

consists of tall columnar cells (fig 4*b*). These explants are maintained in suspension cultures for 15 days. During this time the explants become encapsulated (fig 4*c*) with epithelial cells migrating from the glands (fig 4*d*). While in suspension culture, the epithelial cells also begin to accumulate on the surface of the explant in the form of "blebs" (fig 4*e*). The explants are then placed in petri dishes and allowed to attach to the substrate, where the epithelial cells spread out into a monolayer (fig 4*f*). One of the major problems in culturing the prostate has been the growth of fibroblasts from the stroma. By using the above procedure, we can obtain predominantly epithelial cultures. The age of the donor and the type of serum used affect the type of cell selected to grow. When cells from a 6-year-old donor were grown in horse serum epithelial cultures were obtained, but when fetal bovine serum was used the cultures were predominantly fibroblastic. CPE in prostatic outgrowths begin to appear about 6 days after the explants attach to the petri dish surface. The first CPE observed is the appearance of vacuolated polykaryocytes (fig 5*a*). Figure 5*b* shows a polykaryocyte with cytoplasmic bridges (arrows) extending from this cell to neighboring cells. This leads to cell fusion and enlargement of the polykaryocyte. Nuclear inclusions were also observed in the nucleus of these cells. Some of the inclusions stained deeply (fig 5*c*), while others appeared like holes or nuclear scars (fig 5*d*). CPE described above are generally recognized to be virus induced. When polykaryocytes were examined with the electron microscope, they showed a variety of virus-associated cellular changes. The nuclear scars appeared as light areas in the nucleus (fig 5*e*) and contained microfibrils and nucleocapsids (figs 5*e*, 6*a*, and 6*b*). Proliferation of nuclear membrane and thickening of inner nuclear membrane, which are often seen in Epstein-Barr virus-infected cells, were noticed in prostatic cells (fig 6*a*). Intranuclear and intracytoplasmic nucleoids (fig 5*e*) and nucleocapsids (figs 6*a*–6*c*) were also observed. These particles conform to the size of herpesvirus particles. Increased acid phosphatase activity in the cell membrane of prostatic polykaryocytes is also recognized as a virus-induced change. Fibroblastic cultures derived from the prostates showed rounding and clumping of cells (fig 6*d*), another virus-induced CPE. Although it would be premature at this time to suggest that the observed virus-like particles might be causative or contributory to malignancy, the possibility must be considered. Identification and determination of the nature of these particles are the next most important steps.

M. Pollard: I'd like to show some illustrations of the spontaneous adenocarcinomas that we have encountered in our aged germfree Wistar rats (6) which we have now transplanted into conventional randombred Wistar rats and in tissue culture. Table 1 lists data on eight rats with spontaneous prostate adenocarcinomas thus far encountered in Lobund Wistar rats. The youngest tumor-carrying rat was 22 months old, but the remainder exceeded 30 months of age: 35% of our male rats over 30 months of age have developed spontaneous adenocarcinomas of the prostate, and most of them had tumor metastases to the lungs and less frequently to the kidneys, spleens, and livers. The Lobund Wistar germfree rats develop spontaneously benign adenomas of many endocrine glands (7), but the prostate is the first organ with marked evidence of malignancy. The neoplastic cells penetrated the organ capsule, the cells appeared immature and some

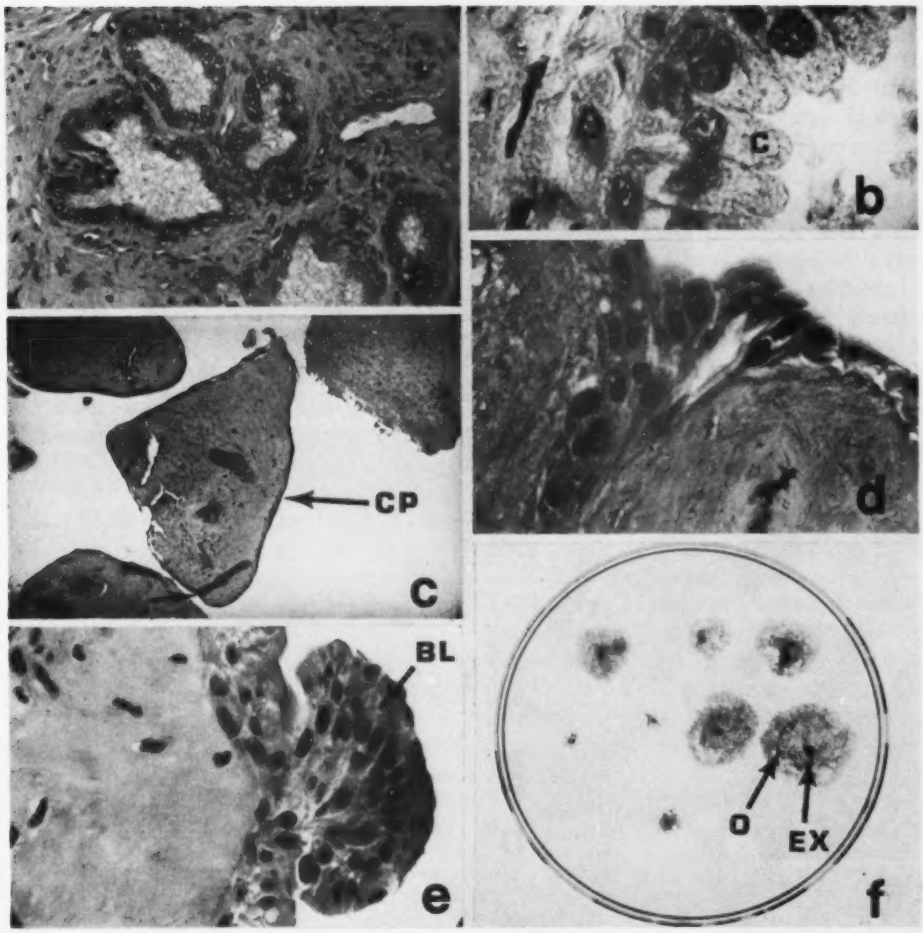

FIGURE 4.—(*a*) Histologic section of a prostatic tissue sample before culture (H & E stain, × 96). (*b*) Prostatic epithelium showing tall columnar cells (H & E stain, × 600). (*c*) Completely encapsulated explant. One gland shows continuation (arrow) of its epithelium with the capsule (CP) (H & E stain, × 24). (*d*) Initial stage of encapsulation involving migration of epithelial cells from a gland to the surface of the explant (H & E stain, × 240). (*e*) Portion of an explant showing multilayered epithelial capsule and a "bleb" (BL) (H & E stain, × 240). (*f*) Seven-day epithelial outgrowth (O) from prostatic explants (EX) (Giemsa stain, × 1).

were in mitosis, and the tumors had metastasized. The tumor cells were transplantable, and after propagation in vitro they reproduced the same tumor in Lobund Wistar rats. Some of the rats with prostatic tumors manifested a myelocyte leukemoid reaction. The related tumors and metastases have areas of necrosis and infiltrations with myelocytes. Seven of the eight tumors were glandular arrangements of epithelial cells (some of them in mitosis) in a stroma of connective tissue cells, collagenous materials, and varying numbers of myelocytes (fig 7). Two such tumors have been transplanted subcutaneously (sc) to Wistar rats. The tumor ulcerates to the surface and spreads through the lymph nodes to the lungs. A second tumor type is predominantly epithelial, with relatively little connective tissue (fig 8). It too is transplantable via the sc route and spreads through lymphatic channels to the lungs. Both cell types

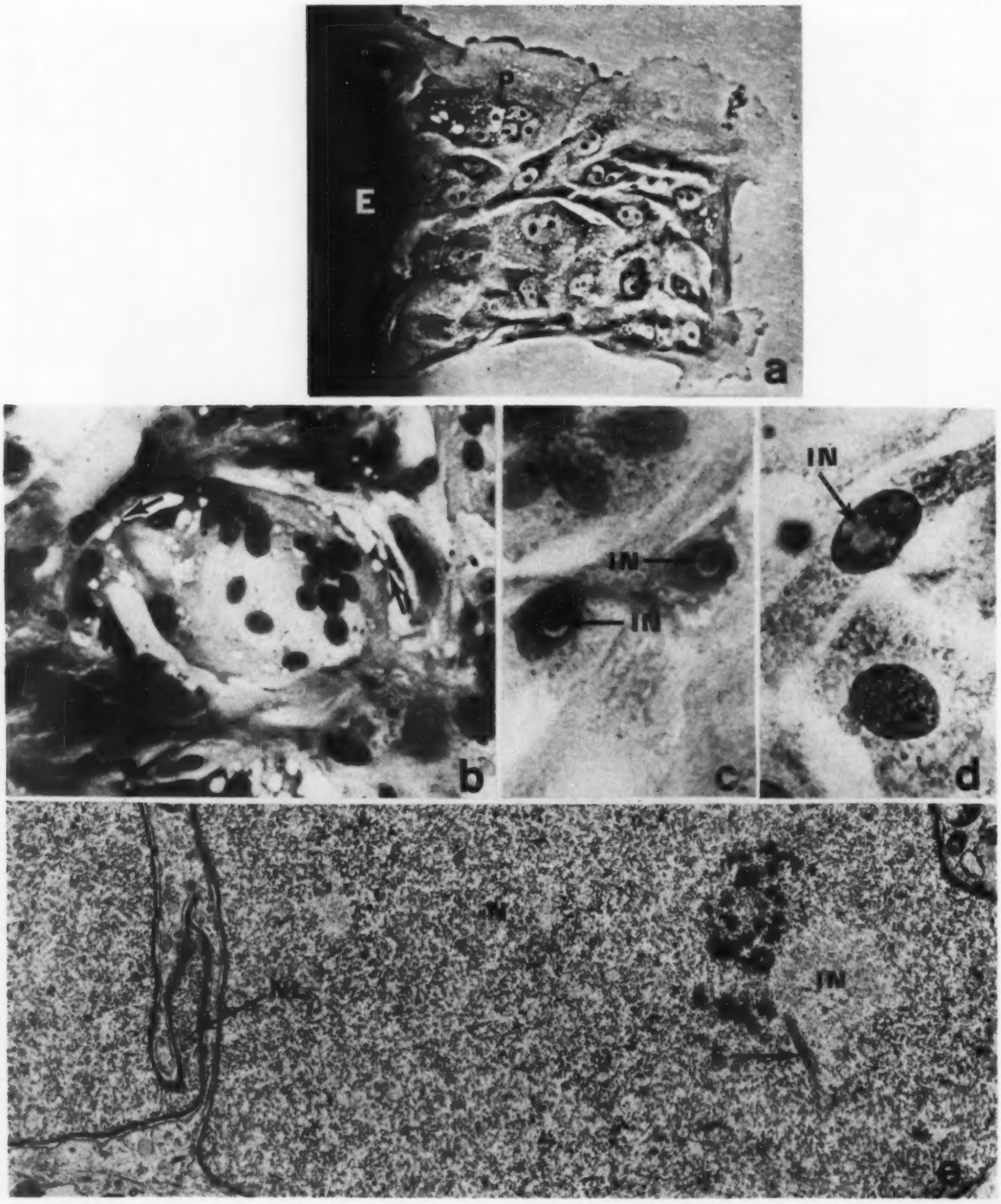

FIGURE 5.—(*a*) Epithelial outgrowth from a prostatic explant (E) 6 days after it was placed in culture. Polykaryocytes (P) can be seen at this time (living culture, phase-contrast, × 338). (*b*) Demonstration of the mechanism of polykaryocyte formation by cell fusion. Note cytoplasmic bridges (arrow) between the large

propagate in tissue cultures, and acid phosphatase was demonstrable prominently in the epithelial cells. These tumors of Lobund rats reflect characteristics of prostatic tumors in man, and hopefully they will serve as experimental model systems thereof. Through germfree status, the rats reached an age of increasing susceptibility to prostatic tumors and the resulting tumors were uncomplicated by irrelevant infections from the environment.

R. Gittes: I would like to present our preliminary results of the past 5 months in attempting to culture carcinoma of the prostate ex vivo in nude mice and tissue culture. The work has been done in association with Dr. A. P. McLaughlin and Mr. J. D. Brooks and is summarized in table 2. Of the eight different tumors heterografted as a fine mince in nude mice, four have yielded "growth." So far "growth" has been obtained only in nude mice treated with added testosterone. Grossly, a tumor mass has grown sc on the flank. The histology is intriguing. Growth retrieved and sectioned at 3 weeks (fig 9) and at 6 weeks (fig 10) is contrasted microscopically to the original tumor. The growing tissue contains fibrous stroma. The original tumors do show such glands surrounded by malignant cells. Acid phosphatase stains (fig 11) show a rich uptake by these glands in the nude mouse transplants. It is likely that we are looking at growth of benign prostatic hyperplasia elements only, but this remains to be proven. We have not achieved transfer of the growth into tissue culture (except for fibroblast-like outgrowth), nor have we documented abnormal karyotypes. As shown in table 2, we have usually had no difficulty in noting a primary outgrowth of epithelial cells from fragments of carcinoma in tissue culture, but we have failed to transfer these as secondary cultures, with or without testosterone in the medium. We have failed to grow anything except fibroblast-like cells from carcinoma of the prostate metastatic to the lymph nodes. I think that "growth" from primary tumors will require critical scrutiny in view of the heterogeneity of the original tissue. It remains to be proven that anyone has grown human carcinoma in secondary cultures. It will require clearcut growth of malignant glands in nude mice or other immunosuppressed hosts, successful passage in tissue culture with chromosomal identification, cloning of epithelial cells, and then repetition of malignant gland formation upon inoculation of the cells into nude mice. That is our goal.

M. Tannenbaum: Figure 12 demonstrates an explant outgrowth pattern from a central core of benign prostatic tissue. We always histologically analyze this central core of tissue or supply house tissue, otherwise we will not know what kind of cells we are cloning. We have done extensive transmission and scanning electron microscopy studies on the explant outgrowth. A histologic analysis of these monolayer cultures demonstrates a fibroblastic pattern in continuity with epithelial cells. Electron microscopy demonstrates that these fibroblastic cells form cell attachments with the epithelial cells. These fibroblast-like cells are morphologically very similar to the basal or myoepithelial cells found in prostatic glands. Figure 13 is a histologic analysis of the central core of tissue which gives rise to the explant outgrowth. The one key histologic finding which is found in all of the benign glands as opposed to most of the cancerous glands is the formation of an epithelial capsule over the raw connective tissue. This capsule, either a single or double layer of epithelial cells, forms in 3–5 days. The prostatic cancers will not do this unless there is (*a*) a cribriform pattern, (*b*) a prostatic

FIGURE 5.—Continued

polykaryocyte and adjacent cells (Giemsa stain, × 338). (*c*) Densely staining nuclear inclusions (IN) with a halo (Giemsa stain, × 500). (*d*) Nuclear inclusion (IN) staining lighter than the nucleoplasm and appearing like a nuclear scar (Giemsa stain, × 960). (*e*) Nucleus (N) from a polykaryocyte showing a scar-type nuclear inclusion (IN). Microfibrils (F) are seen in the vicinity of this scar. A portion of the adjacent nucleus has a protrusion containing a group of dense particles which may correspond to viral nucleoids (NL) (× 18,500).

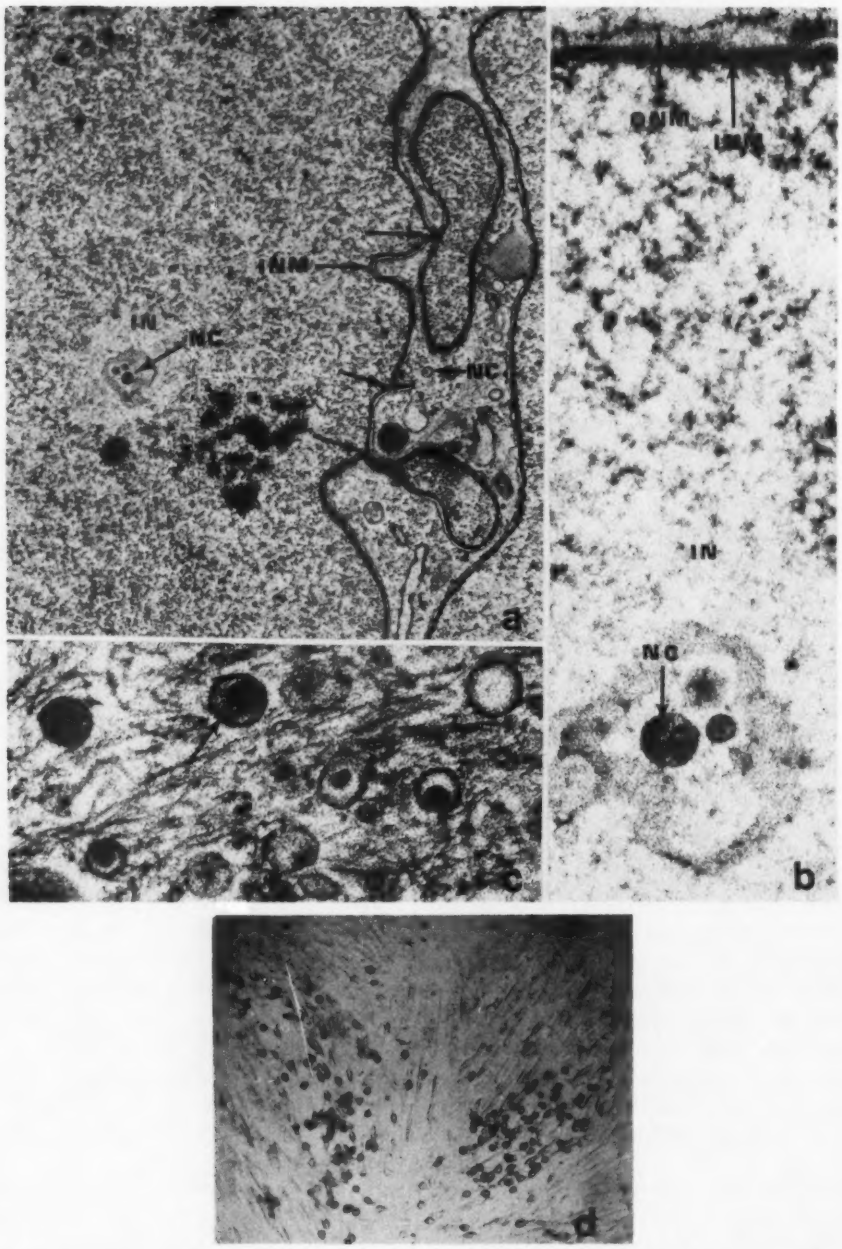

FIGURE 6.—(*a*) Electron micrograph showing changes in the nuclear membrane. The inner nuclear membrane (INM) is thickened, and nuclear membrane shows proliferation and protrusions (arrows). This nucleus also has an inclusion (IN) which contains virus-like particles or nucleocapsids (NC). Another nucleocapsid (NC) is seen in the cytoplasm (× 18,500). (*b*) Portion of the nucleus in fig 4*b* at higher magnification. This shows a nucleocapsid (NC) in the scar (IN). Note a thickened inner nuclear membrane (INM) and relatively nar-

TABLE 1.—Adenocarcinomas of the prostate glands in germfree Lobund Wistar rats

Rat No.	Age (mos)	Weight (g)	White blood cells/mm³	Hematocrit (%)	Spleen weight (g)	Tumor metastases	Leukocytes in tumor
1	22	302	15,700	27	0.31	Lungs	+
2	32	484	26,000	38	1.5	None	+
3*	37	378	—	—	0.75	Multiple organs†	+
4	39	385	71,000	38	1.4	Multiple organs†	+
5	31	381	26,000	24	1.73	Multiple organs†	+
6	35	465	8400	42	1.09	Lungs	+
7	37	388	40,000	27	1.0	Multiple organs†	−
8	38	424	4200	48	0.8	Lungs	+

*Dead on arrival at laboratory.
†Spleen, liver, peritoneum, lymph nodes, and lungs.

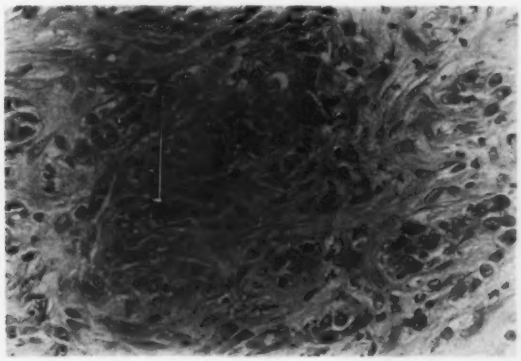

FIGURE 7.—Spontaneous prostatic adenocarcinoma in Lobund Wistar rats. Note arrangements of epithelial cells in a stroma of connective tissue cells, collagenous materials, and varying numbers of myelocytes.

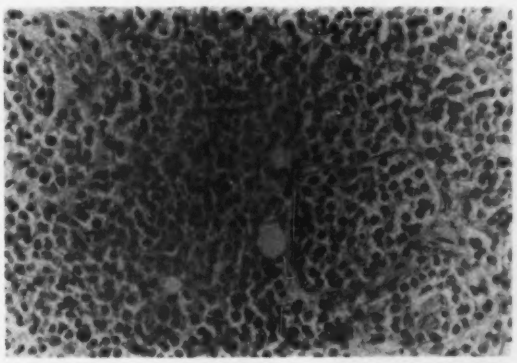

FIGURE 8.—Spontaneous prostatic adenocarcinoma in Lobund Wistar rats. Note predominance of epithelial cells with relatively little connective tissue.

FIGURE 6.—Continued

row outer nuclear membrane (ONM) (× 88,500). (*c*) Nucleocapsids (one indicated by an arrow) in the cytoplasm of a polykaryocyte. Some particles have dense cores, while others show a hollow capsid (× 88,500). (*d*) CPE consisting of round, refractile cells forming foci in a fibroblastic culture derived from benign prostatic hyperplasia (living culture, phase-contrast, × 135).

TABLE 2.—Attempts at ex vivo culture of prostatic carcinoma*

Source	No.	No. of transplants to nude mice	No. of mice with growth	No. of tissue culture attempts	Subculture established
Radical prostatectomy	17	8	4	16	No
Positive lymph node	5	1	0	5	No
Transurethral resection chips	7	1	0	7	No
Needle biopsy	6	0	0	6	No

*Attempts made from Jan 1, 1974, to June 30, 1974.

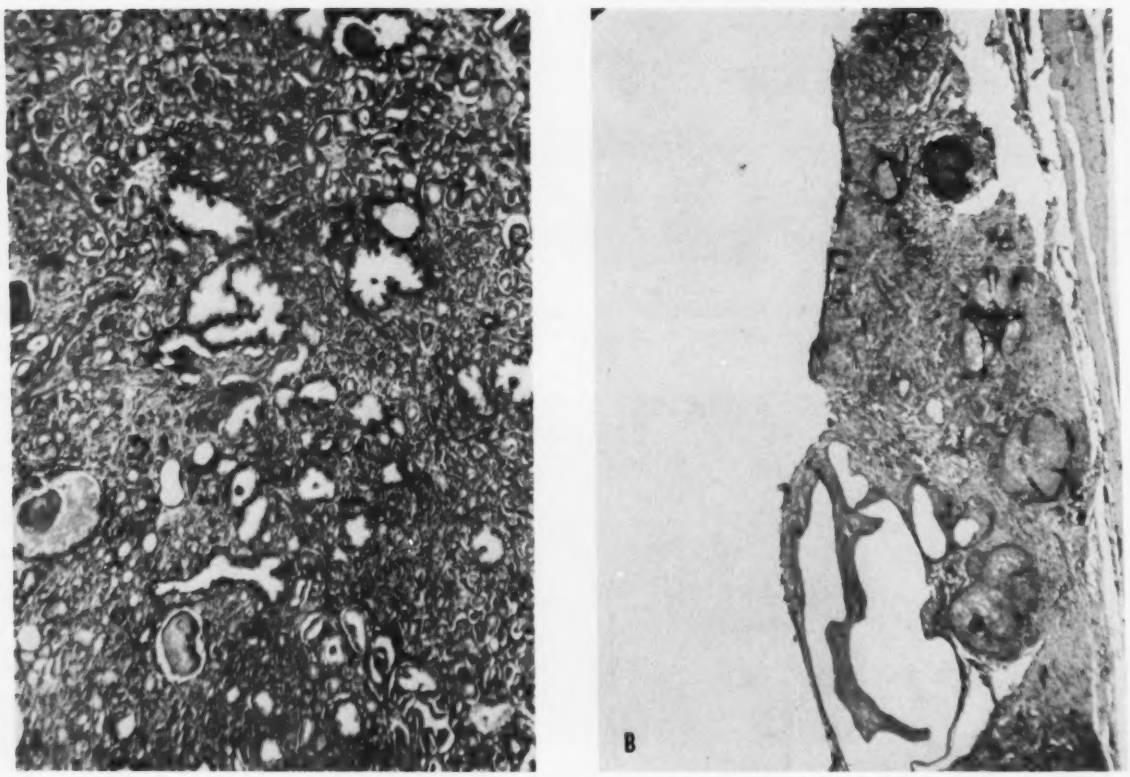

FIGURE 9.—"Growth" from heterografts of prostatic carcinomas in nude mice (A) contrasted with the original tumor (B). Retrieved and sectioned at 3 wks.

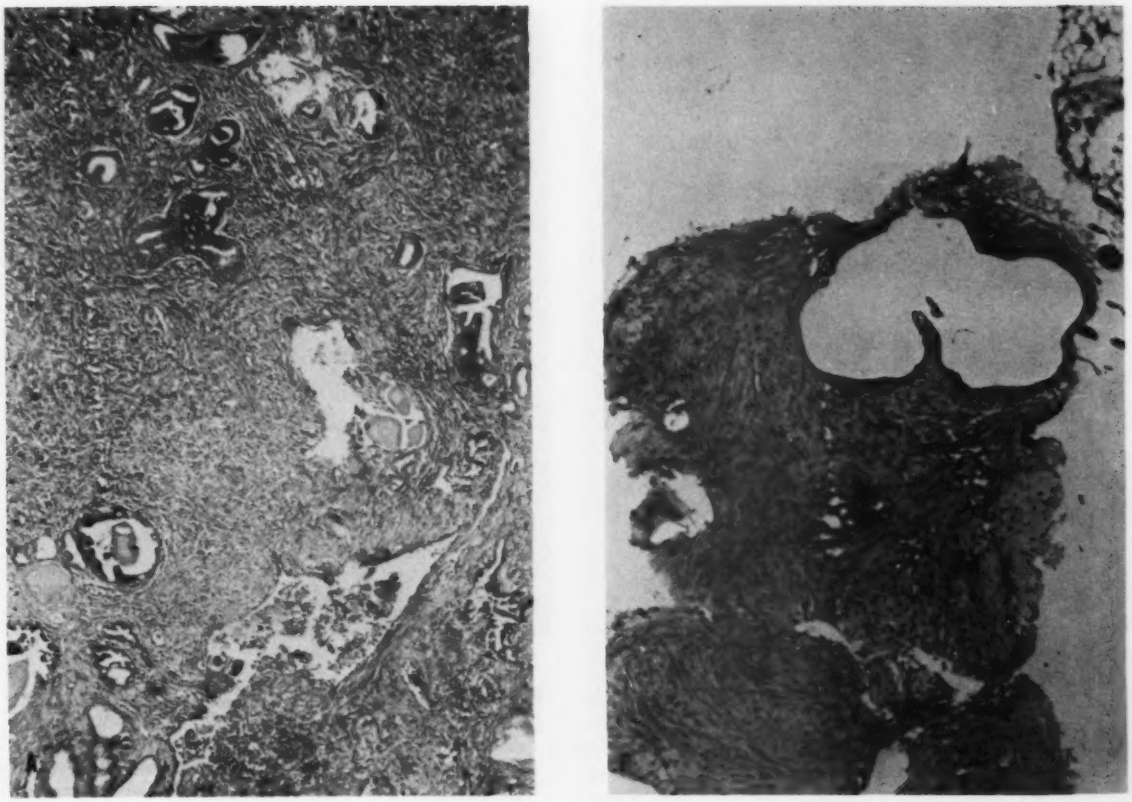

FIGURE 10.—"Growth" from heterografts of prostatic carcinomas in nude mice (A) contrasted with original tumor (B). Retrieved and sectioned at 6 wks.

cancer of the periurethral ducts, or (*c*) an endometrial cancer of the prostate (8). Figure 14 shows a monolayer culture from the eighth subpassage, a low-passage cell line so far, demonstrating a tendency towards differentiation and forming prostatic secretions. These cells are acid phosphatase positive by two different histochemical techniques. Figure 15 is an electron micrograph of the capsule formation found when the cultures are set up for explant outgrowth as well as for organ culture. These basal cells will usually form basement membranes by the seventh to the ninth day of culture. When it breaks out of tissue architecture, prostatic cancer will not form basement membranes visible on electron micrographs. The surface cells of the double-layer capsule are acid phosphatase positive, whereas the basal cells are generally negative. In the center of the central core of tissue there is extensive muscle and fibroblastic degeneration. In cell cultures from several different cell lines (six lines in their sixth to 11th subpassage) that are immunologically positive for herpesvirus types I and II, we have found tubular structures in the cisternae of the rough-surfaced endoplasmic reticulum. They measure 40 nm in diameter. We have not seen budding. We also noted structures similar to those described by Dr. Dmochowski in 1967 (9) but in the benign glands near carcinomatous glands. In our cell lines, which were immunologically free of herpes simplex virus types I and II, we did not find these structures. We have received the antiserum to types I and II from Dr. Burge Hampar at the National Cancer Institute. The

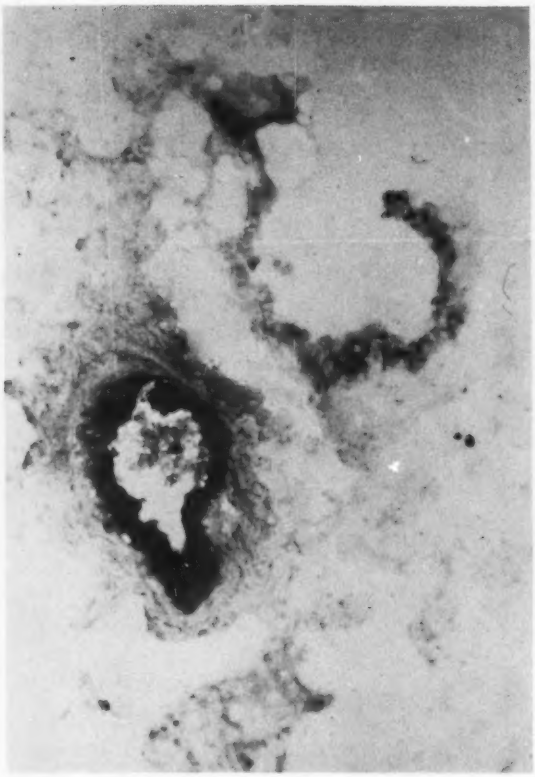

FIGURE 11.—Acid phosphatase stains showing a rich uptake by the glands in the nude mouse transplants.

cell lines we have were stained with this fluoresceinated antiserum which stained the cells in the perinuclear region. Dr. K. C. Hsu in my laboratory has not only fluoresceinated the above antiserum but has also attached ferritin to it. We will also study these cells by means of immunoelectron microscopy to see what structures in the cell are immunologically related to herpes simplex virus types I or II. Unfortunately, the available viral antiserum is against several different components of the herpesvirus and is not directed against any specific component of the virus. The cell lines that we have are also negative to cytomegalovirus as tested by immunofluorescent methods. With these techniques of immunofluorescence and immunoelectron microscopy we can obtain precise localization not only of viruses but also of several other substances such as prostatic

FIGURE 12.—Scanning electron micrograph of benign human prostatic tissue with explant outgrowth. Note stellate cells over surface of central core of tissue as well as over the surface of the petri dish. These cells are attached to each other in most instances

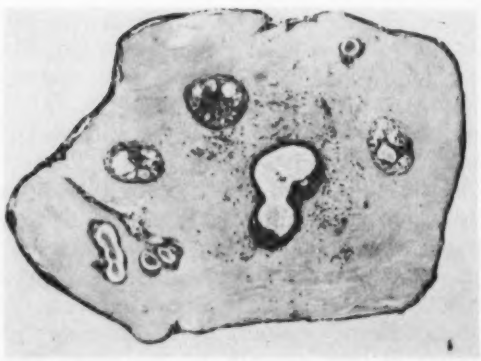

FIGURE 13.—Light histology of central core of tissue which gives rise to explant outgrowth (15 days in culture). Note prostatic epithelial capsule covering degenerated connective tissue and metaplastic glandular elements (× 148).

FIGURE 14.—Electron micrograph of part of explant outgrowth and epithelial capsule. Note partially intact basement membrane formation in lower right-hand corner. Cells also have desmosomes and hence are connected to each other (× 12,500).

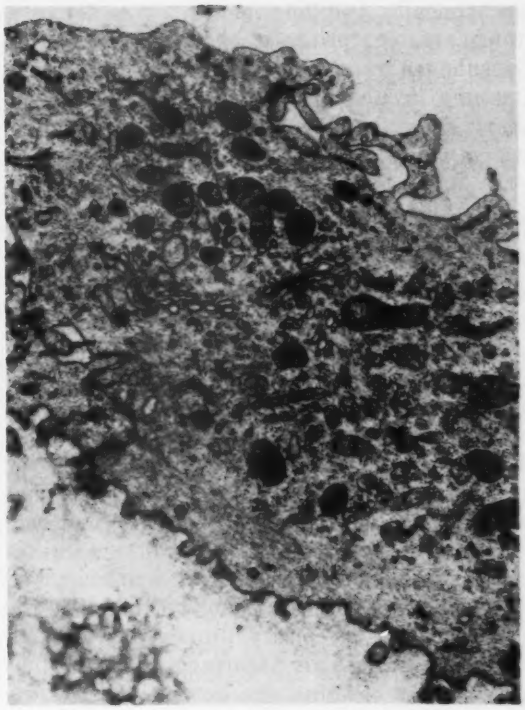

FIGURE 15.—Electron micrograph of subcultured acid phosphatase-positive human prostatic cells. Original source was a carcinomatous prostatic gland. The cells from this 8th subpassage were immunologically positive for herpesvirus type II and other immunoglobulins. Note within the cisternae of the cytoplasm of these cells tubular structures which have substructure at higher magnification (× 27,000).

acid phosphatase, hormone receptor proteins for androgens, or the trophic hormones of the pituitary. To date, we have not been able to subculture many prostatic cancers as monolayer cell cultures unless they were of the above-mentioned three types (cribriform, periurethral ductal, or endometrioid). We have not been able to maintain prostatic cancer organ cultures beyond the fourth day unless there is a variety of hormonal supplementation to the feeding solutions. When this is done the cultures maintain ultrastructural integrity until Day 11. When there is explant outgrowth from the cancers the cells that grow out from the cancer are almost always of a basaloid nature with the above three types of cancers giving rise to these cells. When tissue is taken from a prostatic cancer area, is placed in culture, and produces an explant outgrowth, it doesn't mean that it is cancer. When the central core of tissue is histologically examined, you will often find that there are numerous normal glands mixed in with the tumor. These normal glands are both contiguous and continuous with the explant outgrowth. The cancer is degenerated unless it is of the above three types. As a surgical pathologist I am curious as to whether anyone has a bonafide line or can establish a histologically verified line of prostatic carcinoma in monolayer culture.

W. Voigt: Although spontaneous prostatic tumors in the rat are indeed a welcome and most promising tool, they are not new. We have

just published an article (10) in which we biochemically and hormonally characterize a transplantable prostatic adenocarcinoma in Copenhagen rats first observed by Dr. W. F. Dunning in 1963. I hope to have a chance to show slides of our work with this tumor system.

M. Kaighn: I would like to thank you for the suggestion. We are actually conducting an experiment to test the uptake of tritiated dihydrotestosterone with several of these lines. The other comment I would like to make relates to the expression of specialized properties such as androgen receptors in culture. Whether or not they are expressed depends to a large extent on how we treat the cells. For example, we may have a culture that is derived from liver and does not produce a specific product that we associate with the liver parenchymal cell. If we can change the culture conditions so that this product is made, we are able to study the control of its synthesis. I don't think that the history of tissue culture proves that the environment necessarily permanently affects what a particular cell line can do. We musn't lose sight of that point.

W. Panko: As an answer to Dr. Kaighn's request for a way of verifying that cells grown in culture are indeed prostatic, I would like to suggest the use of the specific binding of a testosterone metabolite, dihydrotestosterone. Virtually no cells outside the male reproductive tract will specifically take up dihydrotestosterone and bind it to a cytoplasmic protein. Even cells which have a demonstrable androgen effect, such as muscle or liver cells, lack the ability to bind specifically dihydrotestosterone. They probably use other metabolites, and I would think that at least in the beginning stages of this work a cell which does not bind dihydrotestosterone and is therefore androgen insensitive, even if it is at least prostatically derived, is not one that we would like to start out working with. In addition, I have two brief questions for Dr. Sato. Can the melanoma cells which he says are androgen sensitive be transplanted into a castrated or hypophysectomized animal? Does the resistance of cytochalasin B-resistant mutants, which can be transplanted into nude mice but not into immunologically intact mice, hold up through the transplantation and growth in the nude mice or is this characteristic lost as the tumor growth commences?

J. Horoszewicz: This appears to be of importance in those cell lines which have lost the Y chromosome and in which the androgen receptors function could be changed. Now perhaps Dr. Sato would like to make his comments.

G. Sato: We haven't transplanted the melanoma cells into castrated or hypophysectomized animals. However, we have transplanted them into female animals and here they grow very, very much slower. Concerning the comment about the steroid receptors, these melanoma cells that home in on the testes have testosterone receptors. The suggestion about looking at the properties of cytochalasin B-resistant mutants growing in nude mice was a very interesting one, and although we haven't done it yet we are going to do it soon.

P. Siiteri: I am intrigued with the occurrence of prostatic cancer in aged male rats and am reminded of some similar observations by Dr. Meites at Michigan State, who finds in his colony of female rats a 50% or 60% incidence of breast carcinoma after 2–3 years of age. The point is that there is something else that is in common among these animals other than age, and that is that they are fat. Old rats tend to be obese, and some investigators believe that there is a correlation between obesity and the incidence of breast cancer, at least in Holland. There is clearly a very strong correlation between obesity and the incidence of endometrial carcinoma. I wonder if someone can say anything about the absence or presence of a correlation between obesity or adipose tissue distribution and the risk of prostatic cancer.

D. Lubaroff: I'd like to ask Dr. Dmochowski a few questions about his immunofluorescence studies (11). Firstly, have you used any of those established epithelial lines to study immunofluorescent-bound antibody? Secondly, you showed high reactivity with MA-160 and HeLa cells. Did you do any absorption studies where you may have absorbed the sera with either

MA-160 or HeLa cells and still maintained reactivity to your fibroblastic cells? Lastly, did you have any sera from the patients from whom the cell lines were derived and did you study their sera to their own tumor cell line?

L. Dmochowski: There was a question by Dr. Hruban which I would like to answer first. These structures that he so beautifully presented to us previously are very pretty electron micrographs. At first I believed that they were virus induced, then I didn't, and now I do believe that they are. I believe this because they have been observed in paramyxovirus infections in human kidneys and they have also been observed in herpes simplex virus infections by one or two investigators. The cell may only have a limited number of reactions in response to a viral infection. I believe that they may indeed be connected with a virus infection but I cannot say at this stage whether it is a paramyxovirus or a herpesvirus infection. I would like to answer all the questions by Dr. Lubaroff but I feel it would be much better if they were answered by my colleague, Dr. Maruyama.

K. Maruyama: I will answer your questions starting from the third point. As Dr. Dmochowski already mentioned, five cell cultures (P-1, P-2, P-6, P-7, and P-9) listed in table 1 of his presentation (11) reacted with their autologous sera. In answer to your second question, we have not absorbed the positive sera with either MA-160 or HeLa cells and retested against the fibroblastic cell cultures. The first question was whether or not we have tested prostatic cancer patient sera in immunofluorescence against the epithelial cell lines as well as the fibroblastic cell lines. Sera tested against epithelial cell cultures and fibroblastic cell cultures were identical. However, as they became available additional sera were tested against some cell lines. Therefore, the number of sera tested against different cell lines varied between 31 and 55.

M. Webber: With regard to the use of the MA-160 cell lines, I have a word of caution. In the past this cell line has been recognized as an epithelial cell line derived from benign prostatic hyperplasia. It has recently been shown by Dr. Walter Nelson-Rees et al (12), using chromosome banding techniques, marker chromosomes, and glucose-6-phosphate dehydrogenase determinations, that MA-160 cells are HeLa cell contaminants. MA-160 should thus be considered a HeLa cell derivative and not a human prostatic cell line until proven otherwise.

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Summary Statement: Etiology and Prevention of Prostatic Cancer

J. S. Horoszewicz [1,2]

I would like to emphasize the value of this session of the workshop for enlarging the scope of the approaches to the study of the etiology of human neoplasms. As we have heard in this session, studies on the etiology of prostatic cancer are not easy. They force the investigators into direct confrontation with human malignancy with much less emphasis on model systems than studies of other groups concerned, for example, with leukemias or breast and skin cancers.

Following such a direct, but more difficult path, requires total mobilization of intellectual and logistic resources, but the possibility of results in the form of findings which are directly and immediately applicable to human disease makes all this worthwhile.

We are still far from reaching ultimate conclusions that have a bearing on our understanding of the etiology of prostatic, as well as other human neoplasms. It is very encouraging, however, to witness such well-charted and diversified approaches shared in a free and exhaustive exchange by so many distinguished scientists. I would like to thank all the speakers and discussants for their contributions and the success of our workshop.

[1] Department of Medical Viral Oncology, Roswell Park Memorial Institute, Buffalo, NY.

[2] *Reprint requests to:* Dr. J. S. Horoszewicz, Roswell Park Memorial Institute, 666 Elm St, Buffalo, NY 14203.

Collection of Postmortem Prostate Tissue Under Sterile Conditions [1]

Theodore I. Malinin [2,3]

Clinical death in man is not accompanied by the concomitant death of all of the organs, tissues, and cells. In fact, the rate of loss of the ability of tissues and organs excised post mortem to function again after either being placed in a favorable laboratory environment or after being transplanted into another host varies considerably between organs and tissues. Based on this gradual cessation of vital functions, a practice of collecting human postmortem tissues for clinical use and laboratory investigation has been established (1,2).[4,5]

Although difficulty in quantitating tissue viability in the laboratory setting is fully realized (3), for the purpose of this discussion the growth of cells in culture is taken as an end point.

Any mention of procurement of tissues from cadavers usually brings forth the question of postmortem bacterial contamination. Some earlier reports in the literature emphasized the frequency of positive bacteriologic findings in the cadavers (4), but others denied rapid postmortem dissemination of the microorganisms (5,6). My experience, gained through the examination of several hundred cadavers, substantiates the latter contention. Similar results, with the exception of skin, have been obtained by other recent investigations (7,8). The purpose of this report is to describe the methods of aseptic collection of cadaveric prostate glands.

MATERIALS AND METHODS

The tissue is collected from the prostate glands of cadavers free of infections and neoplasms and from carcinomas of the prostate glands. The necropsy is performed in a specially devised sterile autopsy suite in a manner previously described in detail (1). Only appropriately gowned and masked personnel are allowed to enter the sterile area which is washed and sprayed with an antiseptic solution after each necropsy or at least once a week.

A general view of the postmortem tissue removal at the University of Miami Tissue Bank is shown in figure 1.

To prevent the introduction of contamination by the wheels of the hospital stretchers on which the cadavers are transported, the stretchers are not wheeled into the operating room. Instead, the stretchers are wheeled to the specially designed window and the cadavers are transferred to the operating table through the window. To cool the body during the tissue removal, a hypothermic blanket is placed on the operating table.

The prostate glands are removed through an anterior abdominal incision. To allow a full exposure, a section of the pubic arch is removed. If the prostate gland is removed together with the bladder neck, the bladder is injected with a strong mixture of antibiotic solutions. Once the prostate gland is removed from the body, it is sectioned in slices and placed in a double sterile container with tissue culture solution. Serum may be added to this solution if desired. The prostatic tissue may be stored in this solution for about 2 weeks at 4–6° C, or it may be frozen with or without cryoprotective agents.

Tissue samples for bacteriologic analysis are taken from each tissue immediately upon removal.

[1] Supported in part by Public Health Service grant CA-15480 from the National Prostatic Cancer Project, National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

[2] Departments of Surgery and Pathology, University of Miami School of Medicine, and the Surgical Service, Veterans' Administration Hospital, Fla.

[3] *Reprint requests to:* Theodore I. Malinin, MD, Department of Surgery, University of Miami School of Medicine, Miami, Fla 33152.

[4] Manual of Procedure for the Storage and Banking of Tissues. Tissue Bank, US Naval Medical School, Bethesda, Md, 1964.

[5] Gresham RB, Perry VP, and Thompson VK. Practical methods of short-term storage of homografts.

FIGURE 1.—A general view of tissue removed at necropsy under sterile conditions.

RESULTS

Prostate glands removed from refrigerated cadavers of all ages retained their capacity to grow in tissue culture for up to 15 hours post mortem. A photograph of cells cultured from an adult cadaver prostate gland is shown in figure 2.

As of this writing, the number of cadavers from which prostatic tissue has been cultivated in vitro has been small (16 cadavers). However, experience gained through the examination of other tissues and organs from 50 cadavers (particularly skin, bone, muscle, and pancreas) indicates a high degree of survival of many postmortem tissues as manifested by their growth in tissue culture.

TABLE 1.—Results of bacteriologic examination of skin and bones from 25 consecutive necropsies

Procurement No.	Age/racial group/sex	Cause of death	Results of bacteriologic examination		Postmortem interval (hrs)
			Skin	Bones	
PS41	50/W/F	Intracerebral hemorrhage	Positive, *E. coli*	No growth	18
PS45	36/B/F	Subdural hematoma	No growth	No growth	7
PS51	34/W/F	Subarachnoid hemorrhage	No growth	No growth	6.5
PS75	46/W/F	Massive cerebral hemorrhage	No growth	No growth	13
PS75	60/W/M	Dehydration	No growth	No growth	24
PS77	60/W/F	Myocardial infarction	No growth	No growth	18
PS79	66/W/M	Right intracerebral hemorrhage	No growth	No growth	10
PS83	65/W/M	Recent coronary thrombosis	Positive, *E. coli*	Positive, *E. coli*	12
PS84	33/W/F	Massive intracerebral hemorrhage	No growth	No growth	11

TABLE 1.—Results of bacteriologic examination of skin and bones from 25 consecutive necropsies—Continued

Procurement No.	Age/racial group/sex	Cause of death	Results of bacteriologic examination		Postmortem interval (hrs)
			Skin	Bones	
PS86	70/W/M	Bilateral pulmonary fibrosis and bilateral confluent bronchial pneumonia	Positive, *Staphylococcus aureus*; coagulase negative	Positive, same organism	20
PS87	86/W/M	Chronic peptic duodenal ulcers with active arterial bleeding	No growth	No growth	21
PS90	63/B/M	Myocardial infarction	No growth	No growth	24
PS91	64/W/M	Cerebral pontine infarction	No growth	No growth	8.5
PS109	60/W/F	Hypertensive cardiovascular disease	Positive, *S. epidermidis*; coagulase negative	Positive, *S. epidermidis*	14
PS110	55/B/M	Multiple pulmonary emboli	No growth	No growth	14
PS111	16/W/M	Head trauma (cerebral edema and multiple fractures)	No growth	No growth	10
PS113	30/W/M	Multiple head injuries	No growth	No growth	10
PS115	48/B/M	Acute myocardial infarction	Positive, *Proteus mirabilis* and *E. coli*	No growth	20
PS116	43/W/M	Primary pulmonary hypertension	No growth	Positive, beta hemolytic streptococci	13
PS119	49/W/M	Subarachnoid hemorrhage	No growth	No growth	6
PS120	67/W/F	Hemispheric infarction	No growth	No growth	4
PS121	70/W/F	Ruptured aortic aneurysm of aortic arch	No growth	No growth	6.5
PS122	74/W/F	Arteriosclerotic heart disease	No growth	No growth	20
PS123	39/W/M	Subdural hematoma	No growth	No growth	7
PS124	43/W/F	Massive subarachnoid hemorrhage	Positive, *S. epidermidis*	No growth	7

Results of bacteriologic cultures obtained from iliac bones, femurs, and skin of 25 consecutive sterile necropsies are summarized in table 1. The pelvic bones were chosen because of their anatomic proximity to the prostate gland. Results of bacteriologic studies on 16 normal prostate glands removed consecutively at necropsy showed two of the glands to be contaminated. One gland was contaminated with *Staphylococcus epidermidis* and the other

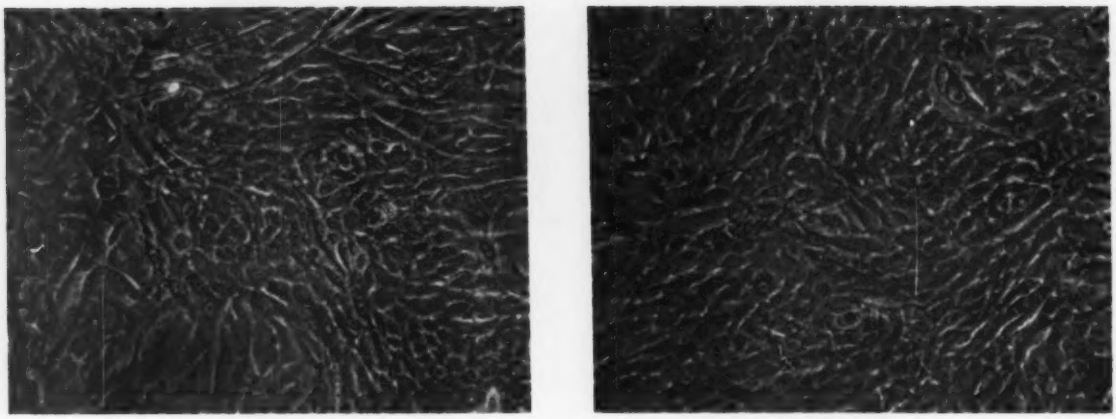

FIGURE 2.—(*Left*) Photomicrograph of in vitro cultured cells from a normal prostate gland of an adult. The prostate gland was removed from a cadaver 16 hours after death. Phase-contrast, × 200. (*Right*) Different field of the same culture.

with *Streptococcus faecalis* and *Escherichia coli*. Contamination in the latter case was attributed to faulty technique. Tissues from two cases of carcinoma of the prostate did not grow microorganisms on cultivation.

DISCUSSION

The brief experience with the postmortem collection of human prostate glands suggests the practicality of using this source of prostate glands for laboratory investigation. This contention is supported by a wider experience gained with other postmortem tissues.

The necessity for using malignant and nonmalignant tissue is self-evident and does not require elaboration. The difficulty of obtaining this tissue, which must be sterile for many laboratory experiments, is also well known. Therefore, it can be anticipated that the establishment of the center for the collection and distribution of prostatic tissue to investigators may, at least in part, alleviate the problem.

The degree of bacterial contamination of many tissues of human cadavers free of frank antemortem infections is not high. This is probably also the case for the prostate gland, but more extensive studies are needed to validate present preliminary observations.

Comparison of bacteriologic cultures of bone and skin from 25 consecutive necropsies showed a somewhat higher incidence of skin contamination as compared to that of bone. Skin is known to have a normal bacterial flora during life (9), and it has been reported that despite standard surgical scrub, sterility of skin is rarely achieved (10). Present experience with postmortem skin, however, does not support the previously reported high incidence of contamination, nor does present experience support the suggestion that half of the "sterile" tissues collected either during surgical procedures or at necropsy will harbor microorganisms (7).

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Tumor Antigen and Acid Phosphatase Isoenzyme in Prostatic Cancer[1]

T. M. Chu, A. Bhargava, E. A. Barnard, W. Ostrowski, M. J. Varkarakis, C. Merrin, and G. P. Murphy[2,3]

SUMMARY

Plasma and prostatic fluid from man, dog, and baboon were measured for carcinoembryonic antigen (CEA) by a radioimmunoassay technique. No CEA was detected in plasma, prostatic fluid, or seminal fluid in 12 dogs and three baboons. Elevated CEA (>2.5 ng/ml) was found in 13 of 20 human prostatic fluids. It was inferred that there was no immunologic cross-reactivity of CEA among man, dog, and baboon. CEA has been isolated and purified from liver tumors. Biochemical studies reveal that CEA consists of 60% carbohydrate and 40% protein. It contains the following carbohydrates: fucose, mannose, galactose, sialic acid, *N*-acetylglucosamine, and a small amount of *N*-acetylgalactosamine. The following amino acids were found in CEA: lysine, histidine, arginine, aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, and cysteine. The amino acid sequence (first 30 amino acids) of the *N*-terminal has been determined. The *N*-terminal amino acid was lysine. Using this study as a model, other tumor antigens from prostatic tumor tissues are being investigated. The acid phosphatase isoenzyme from prostatic tissue was also studied. After a series of purifications, two chromatographic fractions were obtained. Treatment with neuraminidase removed the sialic acid content of the molecule, changed the isoelectric focusing patterns, and abolished the chromatographic heterogeneity. Sedimentation studies indicated a molecular weight of about 100,000. Biochemical studies showed that prostatic acid phosphatase isoenzyme is a glycoprotein which consists of 7% carbohydrate and 93% protein. It contains fucose, galactose, mannose, sialic acid, *N*-acetylglucosamine, and the following amino acids: aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, lysine, histidine, arginine, tryptophan, and cysteine. An antiserum to this purified prostatic acid phosphatase isoenzyme is being prepared in animals.

[Cancer Chemother Rep 59:97–103, 1975]

In investigating the usefulness of the tumor antigens and enzyme assays as markers for the detection and diagnosis of prostatic cancer, we have studied the carcinoembryonic antigen

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[2] Roswell Park Memorial Institute and the State University of New York, Buffalo.

[3] *Reprint requests to:* Dr. T. M. Chu, Roswell Park Memorial Institute, 666 Elm St, Buffalo, NY 14203.

(CEA) and acid phosphatase isoenzyme. Previous reports from this laboratory indicated that patients with active prostatic carcinoma tend to give positive results when tested for CEA whereas patients with inactive disease tend to give negative results. In a series of 28 patients no positive CEA value was detected in any of the 17 patients with inactive disease while seven of 11 with active disease had positive values (1). The difference in these two groups of patients was significant (P value <0.01). Hence, it seems logical to expect that a test based on purified and well-characterized antigens from prostatic tumor tissues may exist and could be used for the detection of prostatic cancer. Since there has been no previous report on prostate-specific tumor antigens, we have employed CEA as a model for our study.

Serum acid phosphatase has been one of the better laboratory tests for prostatic cancer. It is elevated in about 80% of patients with metastases and in about 25% of patients without metastases. An improved enzyme assay which could indicate more sensitively and accurately the course of the disease would be valuable.

This report is a description of our biochemical investigations of the CEA and prostatic acid phosphatase. It is expected that methods derived from this purified and refined reagent could be developed for a better diagnosis of human prostatic cancer.

MATERIALS AND METHODS

Preparation of the enzymes.—Acid phosphatase was isolated from the hypertrophic prostate gland. The tissues were sliced and homogenized in 3 volumes of 0.01% aqueous Tween-80 solution and left in a cold room overnight with stirrings. The supernatant was dialyzed, acidified, and fractionated with ammonium sulfate at pH 4.0. The crude preparation was then chromatographed on Sephadex G-100. The fractions that contained enzyme activities from Sephadex G-100 were pooled and dialyzed against sodium phosphate buffer. They were then chromatographed on a DEAE-cellulose column and fractionated with gradient solution. Two enzyme-active and protein fractions were obtained. These fractions were refiltered on Sephadex G-100, giving a single sharp peak of the enzyme. This material was used for the experiments (2,3).

Assay of acid phosphatase activity.— The enzyme activity was measured using *p*-nitrophenylphosphate as substrate in citrate buffer (pH 5.0, 37°C). The acid phosphatase activity of prostatic fluid was determined by the method of Babson and Phillips (4).

Preparation of the CEA.—Fresh hepatic metastasis from carcinoma of the colon, obtained at the time of autopsy, was dissected free of normal constituents. The tissues were stored at –80°C until the time of extraction. Extraction of tumor tissue was performed essentially as described by Krupey et al (5) and Coligan et al (6). All operations were performed at 4°C. After dialysis and ultrafiltration the extract was lyophilized. The lyophilized material was chromatographed on a Sepharose 4B column using saline buffer (pH 5.5; 50 mM sodium phosphate and 0.1% sodium azide). Fractions were monitored at 280 nm and the CEA activity was determined by radioimmunoassay. The active fractions were pooled, dialyzed against deionized water, and lyophilized. The material was chromatographed on a Sephadex G-200 column until it was homogeneous. All fractions were assayed by Hansen's technique of radioimmunoassay using goat anti-CEA and ^{125}I-CEA from Hoffmann-LaRoche. Radioactivity was determined in a Packard Scintillation Counter (Model 3380) equipped with a gamma spectrometer.

Analyses of amino acids and carbohydrates.—Amino acid analysis was performed on the samples hydrolyzed in 6 N HCl at 110°C for 24 hours under vacuum in a sealed pyrex glass bulb. Beckman Amino Acid Analyzer Model 120B was used. Basic sugars were also determined on the samples hydrolyzed in 4 N HCl at 90°C for 6 hours under vacuum using the same amino acid analyzer. Neutral sugars were determined as alditol acetates by gas chromatography using the method of Lehnhardt et al (7).

Determination of N-terminal amino acid sequence.—The amino acid sequence was determined on the Beckman Protein Peptide Sequencer (Model 890) using the fast quadrol

program. The anilino-thiazolinone derivatives, extracted in the fraction collector, were converted manually to the corresponding phenylthiohydantoin (PTH) derivatives (8). The residues were identified on a Varian Gas Chromatograph (both normal and after silylation [9]). Thin-layer chromatography was performed using two solvent systems (10) and the chromatograms were sprayed with ninhydrin in ethyl acetates. After heating, the residues were identified (11). Further confirmation was carried out wherever useful by hydrolyzing the PTH derivatives with 6 N HCl at 150°C for 24 hours (12) and identifying the free amino acid with an amino acid analyzer.

Immunodiffusion and electrophoresis.—Ouchterlony diffusion was performed using both absorbed and unabsorbed antiserum and the plates were incubated at room temperature under moisture. Immunoelectrophoresis of the purified antigen was performed using rabbit anti-human colonic cancer antiserum using standard techniques.

Specimen collection.—The prostatic fluid collections in dog were made with fistula after the intravenous injection of pilocarpine which has been described in our previous report (13). Human prostatic fluid was collected from patients in the Urology Clinic.

Determination of CEA.—The CEA was determined by a radioimmunoassay technique (14).

Column chromatography, disc electrophoresis, and isoelectric focusing were performed as previously reported (2).

RESULTS AND DISCUSSION

CEA and Acid Phosphatase in Prostatic Fluid

Table 1 shows the determination of prostatic fluid CEA in human patients. If 2.5 ng/ml of CEA was used as the dividing line between normal and abnormal, 11 of the 16 patients with carcinoma of the prostate have elevated CEA in their fluids. The activity of the acid phosphatase was also measured in these 16 patients with prostatic cancer as shown in table 2. An appreciable amount of enzyme activity was detected in each prostatic fluid specimen. However, no positive correlation was found between the elevations of CEA and the activities of acid phosphatase.

TABLE 1.—Determination of prostatic fluid CEA in man

Patient No.	CEA (ng/ml)	Diagnosis
1	81.2	Prostatic carcinoma
2	63.0	Prostatic carcinoma
3	24.0	Prostatic carcinoma
4	20.5	Prostatic carcinoma
5	19.1	Prostatic carcinoma
6	17.9	Prostatic carcinoma
7	16.1	Prostatic carcinoma
8	11.7	Prostatic carcinoma
9	7.1	Prostatic carcinoma
10	6.7	Prostatic carcinoma
11	6.1	Prostatic carcinoma
12	2.2	Prostatic carcinoma
13	1.8	Prostatic carcinoma
14	0.9	Prostatic carcinoma
15	0.1	Prostatic carcinoma
16	0.1	Prostatic carcinoma
17	31.7	Bladder carcinoma
18	12.0	Bladder carcinoma
19	1.2	Renal cell carcinoma
20	1.2	Normal

When the normal prostatic fluid was extracted with saline and gel-filtered on Sephadex G-200, acid phosphatase activity was seen as expected. There were no CEA or CEA-like substance activities observed, as shown in figure 1. On the other hand, the prostatic fluid from cancer patients gave CEA-active fractions as well as acid phosphatase fractions. These results indicated the presence of the tumor-specific antigens from prostate. These an-

TABLE 2.—Measurements of CEA and acid phosphatase in prostatic fluid from patients with prostatic cancer

Patient No.	CEA (ng/ml)	Acid phosphatase level (IU)
1	81.2	8183.6
2	63.0	23.1
3	24.0	56.8
4	20.5	11.2
5	19.1	21.9
6	17.9	122.0
7	16.1	21.6
8	11.7	—
9	7.1	5.9
10	6.7	—
11	6.1	56.0
12	2.2	—
13	1.8	55.7
14	0.9	374.4
15	0.1	13.3
16	0.1	5.4

tigens may be CEA or CEA-like substances (fig 2).

In an attempt to establish an animal system, CEA activities in animals were investigated in over 50 specimens from 12 dogs and three baboons. No CEA was detected in plasma,

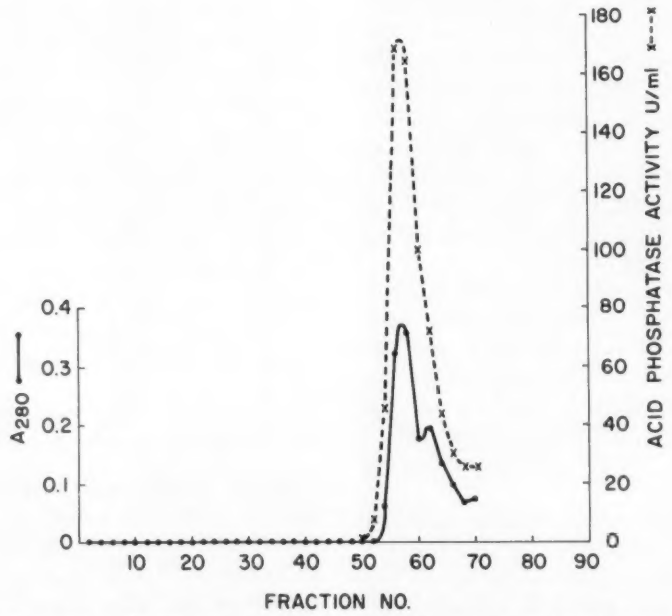

FIGURE 1.—Gel filtration on Sephadex G-200 of prostatic fluid from a normal subject. Fraction volume was 1.2 ml each.

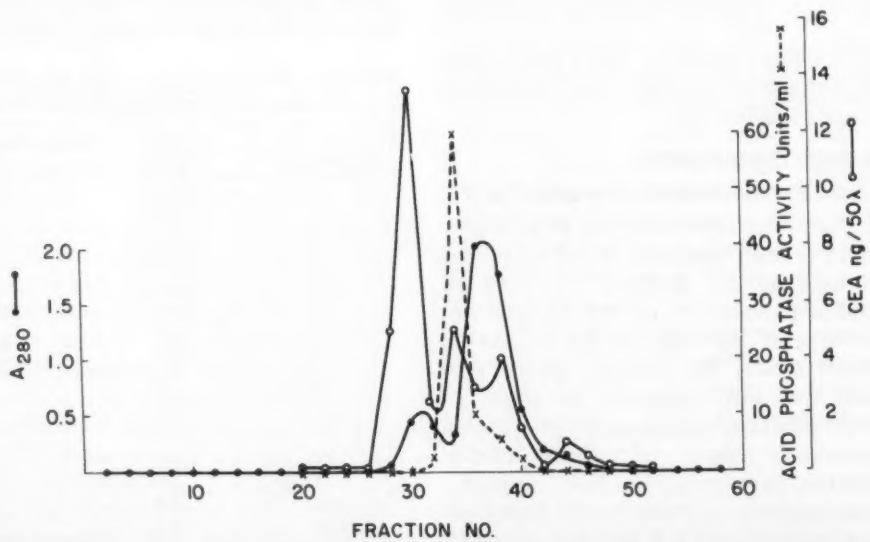

FIGURE 2.—Gel filtration on Sephadex G-200 of prostatic fluid from a patient with prostatic carcinoma. Fraction volume was 2.0 ml each.

prostatic fluid, or seminal fluid in these specimens. It was inferred that there may be no immunologic cross-reactivity of CEA among man, dog, and baboon.

Characterization of Prostatic Acid Phosphatase

As reported previously, two active enzymes and protein fractions were obtained after DEAE-cellulose chromatography with gradient solutions (3). The specific activity of the enzyme was much higher in the first fraction. When put on disc electrophoresis at pH 4.0 and 8.5, the crude extract preparations also showed electrophoretic heterogeneity. The fraction migrating faster towards the anode contained more than 80% of the enzyme activity.

After refiltration on Sephadex G-100, the peak from DEAE-cellulose columns gave a homogeneous peak. During isoelectric focusing these two purified enzyme preparations each gave several peaks. The enzyme preparation with the higher specific activity showed isoelectric points at a higher pH, 4.8–5.2, whereas the second enzyme preparation showed several peaks with isoelectric points at pH ranging from 4.1 to 4.6. Therefore, the heterogeneities of the enzyme preparations were also revealed by the isoelectric focusing technique.

When neuraminidase was employed to remove the sialic acid contents of the enzyme preparations, the heterogeneities of both enzyme preparations on isoelectric focusing were abolished and a single symmetric peak at pH 6.15 was obtained. The removal of sialic acid from the enzyme preparations did not alter the enzyme activities.

The molecular weights of the purified enzyme preparations were determined by the sedimentation equilibrium dialysis technique. The prostatic acid phosphatase was shown to be homogeneous at pH 5.5 and the molecular weight was found to be 100,000. At higher or lower pH, dissociation took place and two subunits were obtained without the cleavage of the disulfide linkages.

The carbohydrate analysis of prostatic acid phosphatase is revealed in table 3. About 7% by weight of the acid phosphatase was composed of carbohydrates. Fifteen residues of neutral sugars, fucose, galactose, and mannose and six residues of sialic acid were in this molecule as well as 13 residues of *N*-acetylglucosamine. No appreciable amount of *N*-acetylgalactosamine was found. The other 93% of acid phosphatase was composed of proteins (18 amino acids) (table 4). The amino acid composition shows a typical pattern for glycoproteins with a very high content of glutamic acid, aspartic acid, threonine, and serine. Large amounts of sulfur-containing amino acids such as methionine and cysteine were also detected.

TABLE 3.—Carbohydrate analysis of prostatic acid phosphatase

Carbohydrate	Content (g/mol)	Mols/mol of enzyme (102,000 molecular weight)
Fucose	346.2	2.1
Galactose	628.0	3.5
Mannose	1728.5	9.5
Sialic acid	1985.0	6.4
N-acetylglucosamine	2280.0	12.7
Total	6967.7	34.2

TABLE 4.—Amino acid composition of prostatic acid phosphatase

Amino acid	Residues/molecule
Aspartic acid	54
Threonine	50
Serine	54
Glutamic acid	100
Proline	50
Glycine	42
Alanine	27
Valine	34
Methionine	20
Isoleucine	27
Leucine	93
Tyrosine	43
Phenylalanine	32
Lysine	45
Histidine	26
Arginine	33
Tryptophan	18
Cysteine	16
Total	764

Characterization of CEA

Perchloric acid-soluble antigens were isolated by chromatography on Sepharose 4B column and Sephadex G-200 column. Two peaks containing CEA-like activity were observed on Sephadex G-200 chromatography. The first peak contained the major activity while the second peak had less activity suggesting the existence of at least two molecular forms. The first peak was repeatedly chromatographed on Sephadex G-200 column until it was homogeneous; this peak was used for our investigations. Both immunoelectrophoresis and immunodiffusion gave a single precipitation line, showing it to be a single species.

The amino acid composition (table 5) showed CEA to be a typical glycoprotein with relatively large amounts of aspartic acid, glutamic acid, serine, and threonine. These may very well serve as the branching points for sugar chains. The composition is remarkable for the absence of methionine and is quite similar to other reports. A significant amount of cysteine was determined in our analysis.

The carbohydrate content of the molecule was determined to be approximately 60% of the whole molecule. A large amount of *N*-acetylglucosamine was obtained. These results would indicate a highly branched structure. Neutral sugars were in the same range as reported by other workers. Differences in sialic acid content may result from differences in the extraction and analytic procedures employed for their determination (table 6).

TABLE 5.—Amino acid composition of CEA

Amino acid	Mols/100 mols
Lysine	3.4
Histidine	1.9
Arginine	3.3
Aspartic acid	12.6
Threonine	9.3
Serine	9.5
Glutamic acid	9.7
Proline	8.2
Glycine	5.6
Alanine	5.9
Valine	6.3
Methionine	0.3
Isoleucine	4.8
Leucine	7.5
Tyrosine	4.4
Phenylalanine	3.9
Cystine/2	2.9

Lysine was found to be the *N*-terminal amino acid as determined by the Beckman Sequencer. The results of sequence will be published elsewhere.

At the present time, we are isolating antigens from prostatic tumors to compare them with the other isolated antigens.

TABLE 6.—Carbohydrate analysis of CEA

Sugar	% Weight of CEA weight
Fucose	8.8
Mannose	10.8
Galactose	17.5
Sialic acid	2.7
N-acetylglucosamine	19.2
N-acetylgalactosamine	<1.0

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Investigation of Specific Antigens in Prostatic Cancer [1]

Charles W. Moncure, Charles L. Johnston, Jr., Warren W. Koontz, Jr., and M. J. Vernon Smith [2,3]

SUMMARY

Rabbits immunized with normal human prostatic homogenates have been shown to develop antibodies specific for one form of prostatic acid phosphatase and a protein antigen which does not appear to possess enzymatic activity. Antibodies were also developed against several other proteins, esterase, two aminopeptidases, and a second form of acid phosphatase. Extensive absorption of the antisera with gluteraldehyde-polymerized serum and kidney homogenate removed all antibodies except those which react with the specific acid phosphatase and the specific protein. These two antigens have been separated from each other by ion-exchange chromatography. The partially purified acid phosphatase has been used to produce antiserum employed for the detection of prostatic cancer in bone marrow aspirates.

[Cancer Chemother Rep 59:105–110, 1975]

The secretory epithelial cells of the prostate exhibit many highly specialized biochemical functions essential to reproduction and subject to hormonal regulation. Many of the pathways involved in the function and regulation of the prostate are still poorly understood; however, it is clear that when prostatic epithelial cells undergo malignant change some of their specialized metabolic and regulatory mechanisms persist in the neoplastic cells. Indeed, the persistence of such specialized features as acid phosphatase production and androgen dependence provides us with valuable diagnostic and therapeutic tools.

Numerous reports (1–4) indicate that one or more unique prostatic constituents are also antigenically specific and can thus be identified immunologically. The current study was undertaken to extend our knowledge of prostate-specific substances through immunologic techniques and to employ specific antisera to study the persistence or alteration of specific substances in prostatic carcinoma.

MATERIALS AND METHODS

Production of Screening Antisera

Initial antisera were obtained by immunizing New Zealand white rabbits with saline homogenates of normal human prostate (and benign prostatic hyperplasia) mixed with complete Freund adjuvant. Normal human tissue was obtained from young adult men who were victims of sudden traumatic death. The tissue was quickly frozen and stored at –85° C. The frozen tissue was then crushed and homogenized with a glass homogenizer in 2 volumes of saline. Each rabbit received 0.5 ml of homogenate and 0.5 ml of adjuvant subcutaneously in two sites weekly for 16 weeks.

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[2] Department of Pathology, Medical College of Virginia, Virginia Commonwealth University, Richmond.

[3] *Reprint requests to*: Charles W. Moncure, MD, Department of Pathology, Medical College of Virginia, Virginia Commonwealth University, Richmond, Va 23219.

Evaluation of Screening Antisera

Sera obtained weekly by ear vein puncture were screened by Ouchterlony (5) immunodiffusion in 2% agar in phosphate buffered saline. A standard Gilford punch was used to cut 3.0-mm wells in a 2-mm layer of agar. Antisera were reacted with normal human serum and a battery of normal tissue homogenates. Tissues used included heart, lung, liver, spleen, kidney, breast, thymus, small bowel, and striated muscle.

Diffusion was carried out for 24–48 hours at room temperature or at 4° C. The plates were then washed in water for 24–72 hours. Various combinations of incubation times, temperatures, and washing times were employed to insure maximum detection of antibodies and minimum loss of enzymatic activity in the tissue extracts.

After washing, the plates were stained for protein, acid phosphatase, esterase, leucine aminopeptidase, β-galactosidase, and peroxidase as follows:

Protein.—The agar was dried and covered with a solution containing 1% amido-black in an aqueous solution of 5% acetic acid and 5% mercuric chloride for 3–5 minutes. The stained plate was then washed in 3% acetic acid.

Enzyme reactions.—Wet gels were covered with freshly prepared chromogenic enzyme stains and incubated until clear staining of precipitin bands occurred. If no staining occurred in the precipitin bands the reaction was continued until background staining around the well occurred. The following enzyme stains were used:

1. Acid phosphatase: 20 ml of 0.1 M citrate buffer (pH 4.8), 20 mg of naphthol AS-MX phosphate, and 30 mg of fast red TR salt. Staining time was 30 minutes (prostate) to 12 hours (negative tissues).
2. Esterase: 20 mg of alpha naphthyl acetate dissolved in 0.5 ml of acetone, then diluted to 20 ml with 0.1 M tris-HCl buffer (pH 7.5–8.0). Add 30 mg of fast red TR salt. Staining time was up to 1 hour.
3. Leucine aminopeptidase: 20 mg of L-leucyl-β-naphthylamide hydrochloride dissolved in 0.1 M tris-HCl buffer (pH 7.5–8.0). Add 30 mg of fast garnet GBC salt. Staining time was 1–2 hours.
4. Peroxidase: 20 mg of benzidine dihydrochloride dissolved in 20 ml of saline. Gels were incubated at room temperature for 1 hour and were then placed in 0.5% hydrogen peroxide.
5. β-Galactosidase: 2 mg of 5-brom-4-chloro-3-indolyl-β-D-galactoside in 5 ml of 0.1 M citrate buffer (pH 4.8). Staining time was overnight (12–18 hours).

Absorption of antisera.—Antisera showing reactions suggesting prostate specificity were absorbed with an immunoabsorbent prepared by polymerizing human serum and kidney homogenate with gluteraldehyde (6). Human serum (19 ml) and homogenate (13 ml) were mixed with 32 ml of 2.5% gluteraldehyde and allowed to polymerize at room temperature for 20–30 minutes. The gel was then put through a stainless steel screen, ground with a glass homogenizer, and washed with phosphate buffered saline. The washed gel particles were incubated overnight in 10% human serum to block unreacted aldehyde groups and were washed again in phosphate buffered saline. Equal volumes of gel particles and antisera were incubated at 37° C for 2 hours followed by overnight incubation at 4° C. The absorbed serum was removed by centrifugation and re-evaluated by immunodiffusion.

Partial purification of prostatic antigens.—Normal prostatic homogenate was centrifuged at 20,000 *g* for 30 minutes in a Beckman Model L5-65 ultracentrifuge with a TI-75 head. The supernate was removed, dialyzed for 72 hours against 3 liters of 0.1 M tris-HCl buffer (pH 8.0) at 4°C, and concentrated on an Amicon μm 10 filter at 4°C. Six ml was applied to a 9 × 15-mm column packed with DEAE Sephadex-A50 in 0.1 M tris-HCl buffer (pH 8.0). The first fraction was eluted with the starting buffer (osmolality, 150 mOsmols, determined by freezing point). The bound fraction was eluted in the starting buffer with a sodium chloride gradient. Osmolality of the eluted fractions was determined by freezing point with a DigiMatic Model 3D Osmometer. The two fractions were concentrated to a final volume of 6.0 ml each and used to immunize another group of rabbits.

Clinical application of anti-acid phosphatase antisera.—Antisera from rabbits immunized with the DEAE fraction containing acid phosphatase have been employed to prepare quantitative immunodiffusion plates and to continue an ongoing study of bone marrow aspirates from patients with prostatic cancer (7,8). The marrow aspirate and a serum sample are placed in two wells cut in an agar gel plate containing anti-prostatic acid phosphatase. After diffusion and washing the plates are stained for acid phosphatase and the diameter of the precipitin ring is measured. A marrow ring which is larger than the serum ring is presumed to indicate metastatic prostatic cancer in the bone (stage IV disease). The immunodiffusion studies are being correlated with microscopic examination of marrow smears with Wright's and acid phosphatase stains.

RESULTS

Initial Immunization and Immunodiffusion

All rabbits immunized with the prostate developed antibodies yielding multiple precipitin bands by immunodiffusion. Initial response was usually detectable within 4 weeks; however, the best results were obtained with rabbits receiving prolonged immunization for 2 or 3 months.

Protein staining (fig 1) demonstrated many precipitin bands, one of which appeared to be prostate specific. All rabbits developed an antibody which produced a large precipitin band with strong acid phosphatase activity when reacted with prostate but no reaction with other tissues (fig 2). Occasionally, a second weak reaction with prostate could be demonstrated near the antiserum well (fig 2). Gels stained for esterase showed a single antiesterase antibody which reacted with identity with all tissue homogenates and weakly with serum (fig 3). No prostate-specific reactions were observed.

Leucine aminopeptidase activity was detected in two prostate precipitin bands (fig 4). One of these gave reactions of identity with a peptidase in kidney and small bowel; however, the second precipitin reaction was only seen against prostate. Occasional immunodiffusion studies resolve this prostatic aminopeptidase into two thin bands of activity. Reactions with three

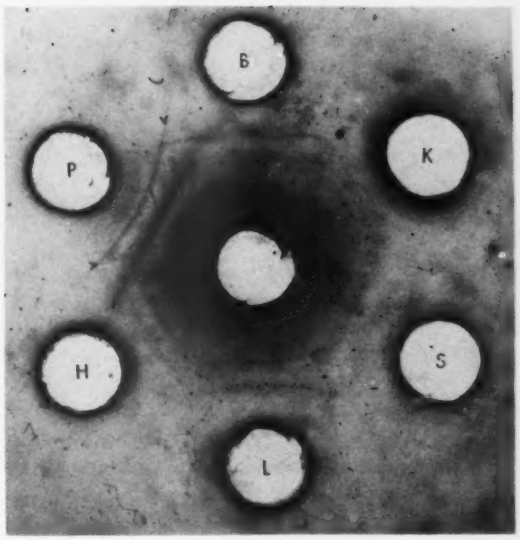

FIGURE 1.—Crude antisera (center well) against human prostate homogenate reacted with prostate (P) and other human tissues. Only the precipitin band marked with the arrow remains after absorption. P = prostate, K = kidney, S = spleen, L = liver, H = heart, B = small bowel.

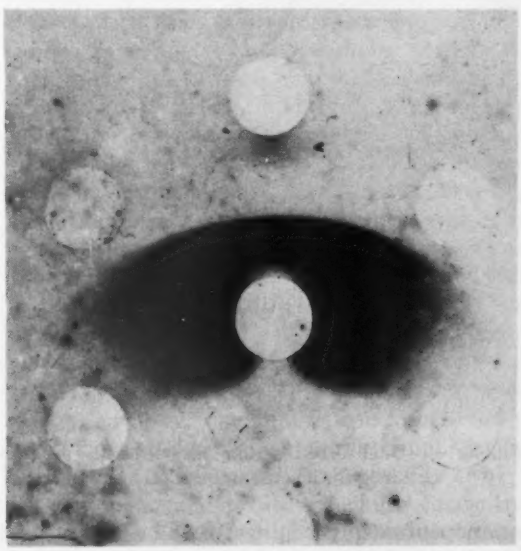

FIGURE 2.—Unabsorbed antiserum against prostate overstained for acid phosphatase to show the faint reaction (arrow) which is removed by absorption.

FIGURE 3.—Unabsorbed antiserum reacted with prostate (P), serum (S), liver (L), kidney (K), small bowel (B), and heart (H). Esterase stain.

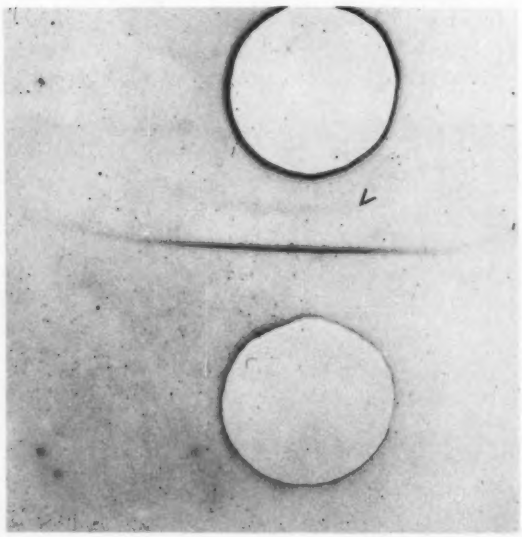

FIGURE 4.—Unabsorbed serum reacted with prostate stained for leucine aminopeptidase.

aminopeptidase precipitin lines have also been obtained with serum from a goat previously immunized with human sperm-free ejaculate (fig 5) (3).

Prolonged staining reactions for β-galactosidase and peroxidase failed to demonstrate any antibodies against these substances, although these enzymes are present in the prostate homogenate.

Absorbed Antisera

After absorption of the antisera only a single faint protein precipitin line against prostate was seen (fig 6). The single strongly reactive acid phosphatase band remained. The second weaker reaction could not be detected.

Anti-leucine aminopeptidase activity was completely removed from most sera by a single immunoabsorption; however, antibodies against the prostatic band sometimes persisted. Anti-nonspecific esterase activity was present but greatly reduced after one absorption of the antiserum. A second absorption removed this antibody.

DEAE Fractionation

The first fraction eluted from the DEAE column with the starting buffer contained the specific protein antigens and the nonspecific esterase. The second fraction eluted from the

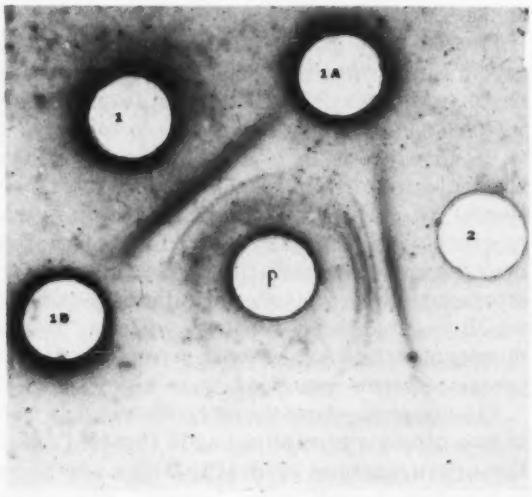

FIGURE 5.—P = prostate. 1 = Rabbit anti-human prostate, unabsorbed; 1A = rabbit anti-human prostate after one absorption; 1B = rabbit anti-human prostate after two absorptions; 2 = goat anti-human sperm-free ejaculate.

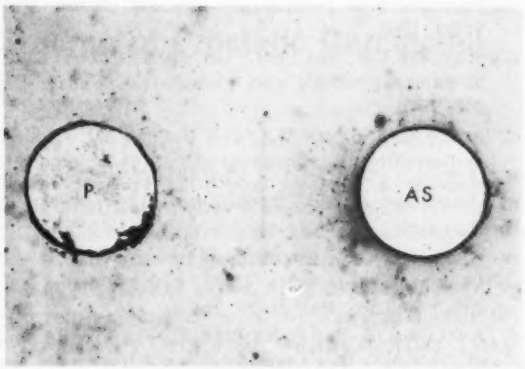

FIGURE 6.—Rabbit anti-human prostate after two absorptions (AS) reacted with prostate (P). Protein stain.

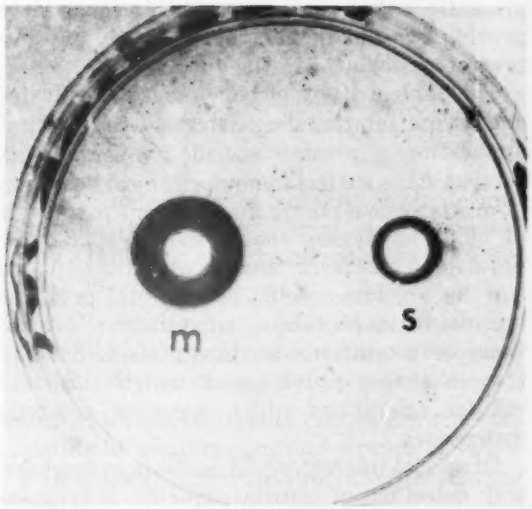

FIGURE 7.—Comparative quantitation of serum (s) and bone marrow (m) prostatic acid phosphatase in a patient with metastatic prostatic cancer.

column at an osmolality of 302 mOsmols and contained acid phosphatase and leucine aminopeptidase activity as well as several protein antigens. Use of absorbed antiserum confirmed the presence of the uncharacterized prostatic antigen in the first fraction and prostatic acid phosphatase in the second fraction.

Clinical Bone Marrow Study

Serum and bone marrow aspirates have been evaluated by comparative immunodiffusion for prostatic acid phosphatase in 145 patients (fig 7). These included patients with prostatic carcinoma, patients with benign or undiagnosed prostatic disease, and control patients with other neoplastic or hematologic diseases. In every case where prostatic cancer cells were demonstrated in the marrow, the immunodiffusion was positive. Four patients with known stage IV prostatic cancer were positive by immunodiffusion although no tumor cells were found in the marrow.

Following prostatic surgery, serum levels may be higher than bone marrow levels. At this point a total of 30 patients have been positive by immunodiffusion. We have not encountered positive immunodiffusion reactions in any patient who did not have proven stage IV prostatic cancer.

The results presented in this paper represent the initial development of antisera to be used in the evaluation of neoplastic dedifferentiation in prostatic cancer. A prostate-specific form of acid phosphatase has been recognized for many years (1–4,9) and previous reports have described a second prostate-specific antigen (3,4) detected only as a protein precipitin band. Our current studies confirm both of these observations and demonstrate that these two antigens can be separated by ion-exchange chromatography. The biochemical characterization of the non-enzymatic prostatic antigen is in progress.

The existence of other prostate-specific substances requires further investigation. Our current data from serum absorption studies suggest that most of the antibodies developed against prostate homogenate show no tissue specificity. Although many protein precipitin bands are obtained with neat serum, only one persists after immunoabsorption with serum and kidney. The esterase and aminopeptidase antibodies also appear to lack prostate specificity, however, the aminopeptidases require much more study. While apparently not specific, one form of this enzyme (and possibly two) is present in normal prostate in high concentration but undetectable in many other tissues. Matilla (10) has reported that this prostatic leucine aminopeptidase is reduced in malignant prostatic tissue. Variations in this and other

prostatic enzymes and specific antigens may provide a means of immunochemically grading prostatic neoplasia in the future.

The clinical study of bone marrow aspirates and serum samples demonstrates one practical application of prostate-specific antisera. In its present stage we feel immunochemical detection of prostatic acid phosphatase in bone marrow is of value in staging the disease. We are now developing antisera and quantitative plates for the prostate-specific protein and prostatic leucine aminopeptidase. Quantitative evaluations of alterations in prostate-specific substances should provide new insight into the cellular alterations which occur in prostatic malignancy.

Of special interest would be the immunochemical detection of prostate-specific substances which regulate hormonal dependence. Testosterone-5α-reductase or the 5α-dihydrotestosterone receptor protein (11) may be such substances. There are no direct immunochemical data on these prostatic constituents at this time; however, reports of experimental atrophic changes in the dog prostate (12,13) after the injection of anti-dog prostate antiserum and clinical remission of metastatic prostatic cancer after cryosurgery of the prostate (14,15) strongly suggest that antibodies may be developed against prostate-specific substances which regulate cellular metabolism and survival. The identification of such specific substances should provide a rational approach for the development of new diagnostic, prognostic, and therapeutic procedures.

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Grading of Prostatic Carcinoma

F. K. Mostofi [1,2]

Generally speaking, three factors affect the prognosis of a neoplasm: the response of the patient, the commissions and omissions of the physician, and the potentialities of the neoplasm. These have been discussed in detail elsewhere (1). As far as the potentialities of the neoplasm are concerned, local extension (staging) as determined by clinical, surgical, and pathologic examination of the primary tumor and the histopathologic appearance of the tumor are the two most important factors. My presentation will deal solely with the histopathology of the tumor. I should like to emphasize that because we have just started our grading project, this report is simply a statement of the rationale and method of approach and not a report of the observations. We felt, however, that a report at this time may stimulate others to test the methods.

Grading of tumors is an attempt on the part of the pathologist to provide the clinician with an estimate of the growth and malignant potential of the tumor. Broders (2) was the first to formalize a system of grading:

Grade I: three fourths of the tumor is differentiated and one fourth is undifferentiated.

Grade II: the proportion of differentiated to undifferentiated components is about equal.

Grade III: three fourths of the tumor is undifferentiated and one fourth is differentiated.

Grade IV: tumor shows no tendency to cell differentiation.

Young and Davis (3) proposed the following system:

Group 1: extra-acinous growth, consisting of a multiplication of small acini, referred to as typical adenocarcinoma of the prostate.

Group 2: extra-acinous growth, newly formed glandular structures being more elongated and branching and the stroma less plentiful than in group 1.

Group 3: intra-acinous growth in which the tumor cells proliferate within preformed acini with no stroma between them.

Group 4: undifferentiated invasive growth, consisting of small masses and strands of tumor cells without lumen pushing into surrounding tissue.

In a detailed analysis of 100 prostatic carcinomas, Evans et al (4) took into consideration eight histologically detectable aspects of carcinoma of the prostate: acinous structure, cell structure, density of cytoplasm, nuclear characteristics, presence of nucleoli, mitotic figures, fibrosis, and inflammation. They devised a composite grade. About two thirds of the cases were grade III and the other third were about equally divided between grades I, II, and IV. They found that patients in grades I and II had longer survivals, less metastatic frequency, and more favorable response to therapy than patients in grades III and IV. This work has not been corroborated.

Muir (5), Kahler (6), and Pool and Thompson (7) claimed that grading of prostatic carcinomas had good prognostic significance. On the other hand, Herbut (8) felt that it was difficult to grade carcinomas of the prostate. At about the same time, I reviewed 100 prostatic carcinomas and found a wide range of histologic patterns which made it difficult if not impossible to grade them. Many pathologists had the same experience and refused to grade these carcinomas. Others, however, did grade them but often included as grade I, carcinomas that were incidentally found in prostates removed for hyperplasia. This was inconsistent because grading has nothing to do with whether or not a tumor is clinically detected.

Beginning in the early 1960s there was a

[1] US Armed Forces Institute of Pathology, Washington, DC.

[2] *Reprint requests to*: F. K. Mostofi, MD, US Armed Forces Institute of Pathology, Washington, DC 20306.

renewal of interest in grading of prostatic carcinoma. Vickery and Kerr (9) and Jewett et al (10) claimed good correlation between grade and survival. They employed three grades.

In mid-1960 Dr. Byar and I decided to investigate the problem further. We reviewed some 300 prostatic carcinomas from the cases studied by the Veterans' Administration Cooperative Urological Research Group (VACURG). We found that in specimens removed by transurethral resection or prostatectomy, we could not repeat ourselves unless we marked a specific area and went back to that area. We had better success with needle biopsies.

Why is grading of prostatic carcinomas so difficult? It is generally recognized that grading is arbitrary and subjective at best. Certain features of prostatic carcinomas make the problem still more difficult: carcinoma of the prostate is a slowly growing tumor, many have an unpredictable behavior, almost every tumor presents a wide range of cytology and histology, and the criteria for differentiation and anaplasia have not been clearly defined.

Gleason (11) has devised an ingenious method to solve the problem of variations in histologic pattern. He observed that a majority of prostatic carcinomas had either a single pattern or, at the most, two patterns of differentiation: (*a*) a predominant pattern, and (*b*) a secondary pattern. In each category the carcinomas were subdivided into five patterns:

Pattern 1: very well-differentiated, small, closely packed, uniform glands in essentially circumscribed masses.

Pattern 2: similar to pattern 1 but with moderate variation in size and shape of glands and more atypia in the individual cells; still essentially circumscribed, but more loosely arranged.

Pattern 3: similar to pattern 2 but marked irregularity in size and shape of glands, with tiny glands or individual cells invading stroma away from circumscribed masses, or solid cords and masses with easily identifiable glandular differentiation within most of them; may be papillary or cribriform.

Pattern 4: large clear cells growing in a diffuse pattern resembling hypernephroma; may also show gland formation.

Pattern 5: very poorly differentiated tumors; usually solid masses or diffuse growth with little or no differentiation into glands. In cases which showed more than one pattern, the second pattern was classified and recorded.

In subsequent reports, Mellinger et al (12) and Gleason et al (13) have claimed that combining the predominant and secondary patterns with clinical stage of the tumor provided them with a very reliable prognostic index. A few pathologists who have tried Gleason's system have found it difficult to reproduce. We are now seriously testing the classification and I am happy to report that we have not found it an unusually difficult task.

In my analysis of the problem of grading prostatic carcinoma I have been impressed with the fact that in all grading systems, especially in the prostate, the terms "differentiation" and "anaplasia" have not been clearly defined. Pathologists have tended to confuse these terms.

There are three components in the mature adult prostate: the glands, the epithelial cells, and the stroma. In the embryonic stages the prostate is represented as outpocketings of transitional epithelial cell nests from the posterior urethra. As sexual maturation takes place these nests develop to form glandular and ductal structures. Thus, in the normal prostate differentiation consists of formation of glands (and ducts). Tumors that form glands may justifiably be regarded as differentiated neoplasms and those that do not form glands may be regarded as undifferentiated tumors. Differentiated tumors form glands which may be large (fig 1), intermediate (fig 2), or small (fig 3). The glands may be fused (fig 4), have a cribriform gland-in-gland pattern (fig 5), or have a papillary configuration (fig 6). In contrast, in undifferentiated tumors the cells occur singly (in rows or columns) (fig 7) or in sheets (medullary pattern) (fig 8). To avoid confusion I would like to reserve the term "differentiation" for this aspect of prostatic carcinoma.

Usually, the cells lining the normal prostatic acini or those of benign prostatic hyperplasia display a rather wide range of cytology depend-

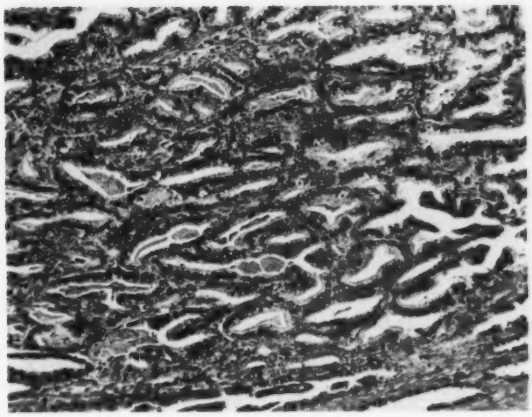

FIGURE 1.—Carcinoma of the prostate forming large glands. (× 115)

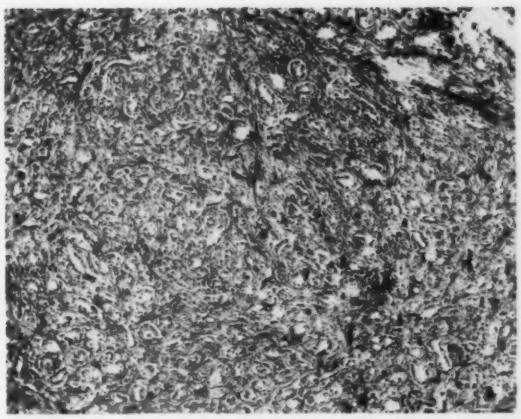

FIGURE 3.—Carcinoma of the prostate forming small glands. (× 50)

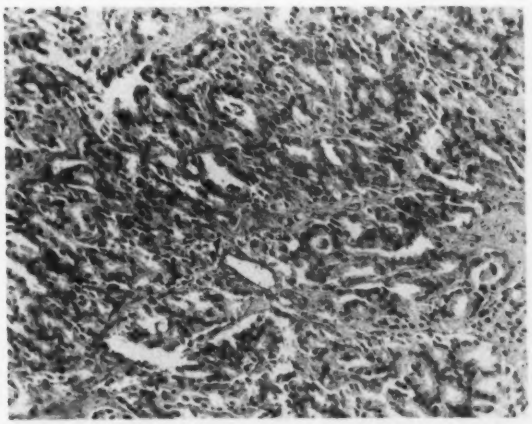

FIGURE 2.—Carcinoma of the prostate forming intermediate-sized glands. (× 150)

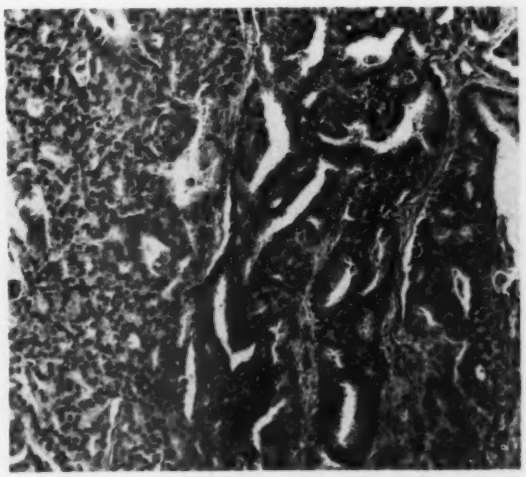

FIGURE 4.—Carcinoma of the prostate showing fusion of glands. (× 200)

ing at least in part on the age of the patient and the functional state of the cell. The cells range from low cuboidal to tall columnar, and the cytoplasm ranges from pale to vacuolated. These differences are reflected in carcinomas of the prostate. In some tumors the cytoplasm is vacuolated, in others it is pale, and in still others it is granular. There is little information about the causes of these variations and we know almost nothing about the prognostic significance of these cytoplasmic variations.

The nuclei of prostatic acini, as well as the nuclei of carcinomas, vary from pale vesicular to hyperchromatic. Many carcinoma cells have a pale or dark-staining nondescript homogeneous nucleus. Others have a vesicular nucleus, some have a coarse distribution of chromatin, and still others have vacuoles. Nucleoli may be absent, small, or very prominent; they may be single or multiple.

Variations from normal in size, shape, stain-

FIGURE 5.—Carcinoma of the prostate showing gland-in-gland pattern. (× 50)

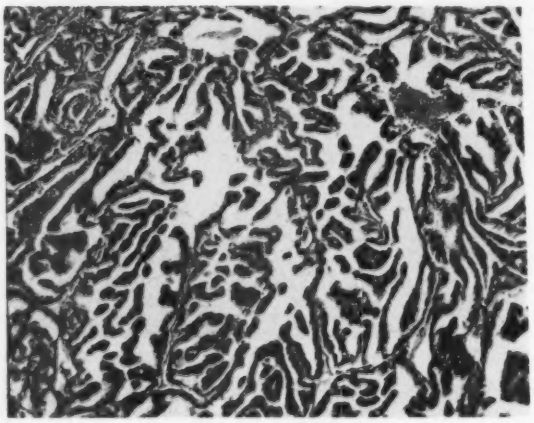

FIGURE 6.—Carcinoma of the prostate showing papillary configuration. (× 50)

ing, and chromatin distribution of nuclei should preferably be referred to as anaplasia. Thus, carcinomas of the prostate may show slight variations from normal: slight anaplasia (fig 9), moderate variation and moderate anaplasia (fig 10), or marked variation and marked anaplasia (figs 11 and 12).

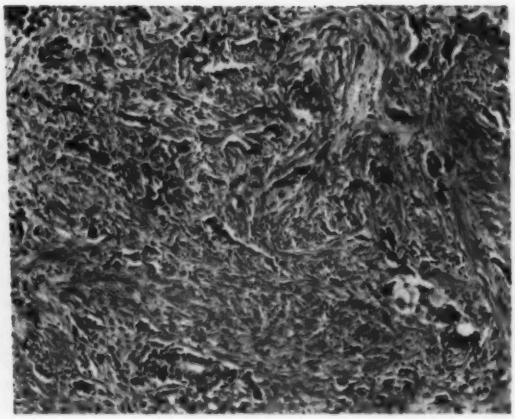

FIGURE 7.—Carcinoma of the prostate in which most tumor cells occur singly in rows or in columns. Some gland formation persists. (× 130)

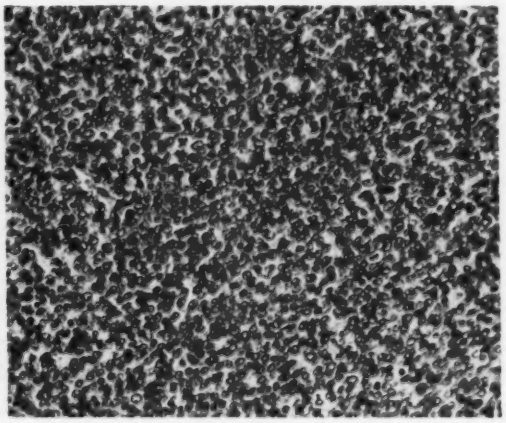

FIGURE 8.—Carcinoma of the prostate showing solid medullary pattern. (× 200)

Combining differentiation with degree of anaplasia we have proposed the following grading system:

Grade I: well-differentiated glands with nuclei that show slight nuclear anaplasia.

Grade II: gland formation but the nuclei show moderate nuclear anaplasia.

Grade III: glands with marked nuclear anaplasia or tumors that are undifferentiated (do not form glands).

FIGURE 9.—Carcinoma of the prostate showing slight nuclear anaplasia. (× 110)

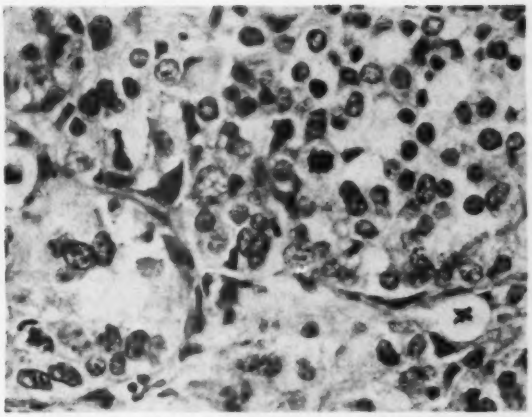

FIGURE 11.—Carcinoma of the prostate showing marked anaplasia. The nuclei vary from small to large and from vesicular to hyperchromatic. Several abnormal mitoses are seen. (× 380)

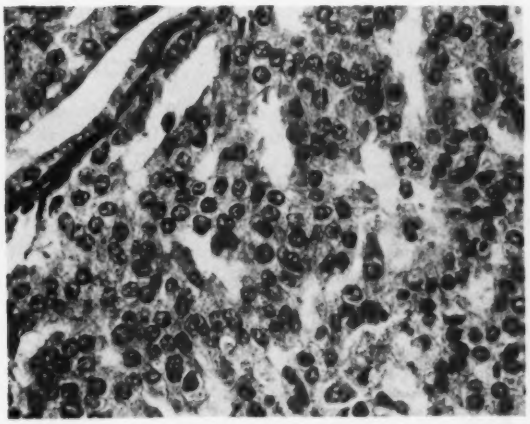

FIGURE 10.—Carcinoma of the prostate showing moderate anaplasia. There is moderate variation in size, shape, and staining of nuclei. (× 450)

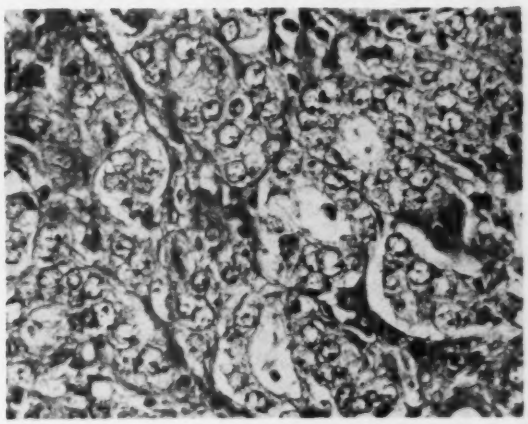

FIGURE 12.—Carcinoma of the prostate showing marked nuclear anaplasia. The nuclei vary considerably in size. There is marked vacuolization of nuclei. (× 450)

Another feature worth investigation is the relationship of the epithelial elements to the stroma. The stroma may be of more or less normal amounts (figs 2, 3, and 5); it may be decreased (fig 1), absent (fig 8), or increased (fig 7).

Except for the previously described work of Evans et al (4) on 100 carcinomas, there has been no systematic review of various histologic and cytologic features of prostatic carcinomas in relation to clinical behavior. In our analysis of cases we have devised a rather detailed checklist (table 1).

To date, we have reviewed two groups of cases. With Professor Schroeder of Wurtzburg, Germany, we have studied 350 prostates from Dr. Elmer Belts' series. A 15-year followup on these patients is available. We have also studied

TABLE 1.—Checklist for histologic study of carcinoma of the prostate

Name of patient _____ AFIP accession No. _____ Hospital _____

Node biopsy ☐ Transurethral resection ☐ Prostatectomy ☐

	Grade
Variation of glands	
None	0
Some	1
Moderate	2
Extensive	3
Glands (dominant and secondary)	
Large	1
Small	2
Micro	3
Fused	4
Glands-in-glands (cribriform)	5
Medullary	6
Columns and cords	7
Papillary	8
Adenoid cystic	9
Endometroid	X
Mucin	
None	0
Small	1
Much	2
Cytoplasm	
Pale	1
Vacuolated	2
Eosinophilic	3
Basophilic	4
Amphophilic	5
Size of cell	
Small	1
Medium	2
Large	3
Size of nucleus	
Small	1
Intermediate	2
Large	3
Variation	
Slight	1
Moderate	2
Marked	3
Appearance of nucleus	
Finely granular chromatin	1
Coarsely granular chromatin	2
Homogeneous nondescript	3
Mitoses	
None	0
Present	1
Nuclear vacuoles	
None	0
Present	1

	Grade
Size of nucleoli	
None	0
Small	1
Medium	2
Large	3
Amount of stroma	
Closely packed glands	1
Normal	2
Increased (desmoplastic)	3
None	4
Relation of tumor to stroma	
Tumor circumscribed	1
Tumor infiltrating	2
Stromal reaction	
None	0
Granulomatous	1
Hyalinized	2
Necrosis	3
Inflammatory	4
Inflammatory cell infiltrate	
In and around tumor	1
Elsewhere	2
Amount of infiltrate	
Slight	1
Moderate	2
Marked	3
Type of infiltrate	
Lymphocytic/plasma	1
Polymorphonuclear leukocytes	2
Eosinophilic	3
Macrophages	4
Amount of tumor	
Small	1
Medium	2
Much	3
Unifocal	4
Multifocal	5
Extension of tumor	
Tumor at surgical edge	1
Tumor surrounded by normal tissue	2
Tumor extending to seminal vesicles	3
Tumor infiltrating capsule	4
Tumor extending to bladder or urethra	5
Tumor infiltrating skeletal muscle	6
Tumor extending to periprostatic tissue	7
Associated lesions	
None	0
Associated with atrophy	1
Associated with hyperplasia	2
Granulomatous reaction	3
Infarction	4

TABLE 1.—Checklist for histologic study of carcinoma of the prostate—Continued

	Grade
Grade of tumor	
Slight anaplasia	1
Moderate anaplasia	2
Marked anaplasia	3
Gleason patterns	
Well-differentiated, uniform, single glands closely packed together in masses with relatively circumscribed boundaries	1
Well-differentiated but more variable single glands, slightly spaced apart, boundaries less well circumscribed	2
Moderately differentiated glands ranging from small to large, growing in spaced-out infiltrating pattern; may be papillary or cribriform	3
Raggedly infiltrating, fused glandular tumor frequently with pale cells, resembling hypernephroma	4
Anaplastic carcinoma with minimal glandular differentiation, infiltrating prostatic stroma diffusely	5

300 cases from the VACURG. The data have not as yet been analyzed but our preliminary impression is that both differentiation and anaplasia are significant in prognosis.

In conclusion, may I say that an attempt is being made to determine whether any one of a number of histologic features of prostatic carcinoma or any combination of these features has significance in prognosis.

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Potential Screening Agents for Prostatic Cancer[1]

Harry E. Hadd [2,3]

We plan to initiate a screening program oriented specifically toward the goal of early diagnosis of prostatic carcinoma in man. This program uses visualization of the human prostate by means of extracorporeal scanning with the Anger scintillation camera after administration of a gamma-emitting compound that mimics the characteristic nuclear chromatin-binding properties of 5α-dihydrotestosterone (5α-DHT; 17β-hydroxy-5α-androstan-3-one).

We propose to determine the biologic profile of a series of compounds related to 5α-DHT and its 19-iodo analogs. We will examine the androgenic profile of these compounds (*a*) by the castrated male rat androgen bioassay, (*b*) by the ability in vitro of the mammalian prostate to exhibit uptake and binding of the radioiodinated analogs, and (*c*) by the in vivo time-tissue distribution in male rats and dogs after administration of the radioiodinated compounds.

If this preliminary investigation shows that one or more of the 19-iodo analogs accumulate in the prostate and a viable target/nontarget ratio is obtained, in analogy to 5α-DHT, this circumstance might be useful in the recognition of early increased androgen-dependent prostatic carcinoma and benign prostatic hyperplasia in man. This could lead to (*a*) the envisioning of bone metastasis specific to the prostatic malignancy, and (*b*) the development and testing of antiandrogens specific to the reduction of the hyperplastic process. Accuracy of staging would be an important ancillary product of this technique.

In the first portion of this investigation we synthesized 19-iodo-5α-DHT. Starting material in the synthetic sequence was either 3β-acetoxy-19-hydroxy-5-androstene-17-one (compound 3) or 19-acetoxy-3β-hydroxy-5-androstene-17-one (compound 2) (fig 1).

Compound 3 was realized through the following set of reactions: the $Pb(OAc_4)$ photolytic activation of the bromohydrin derived from 3β-hydroxy-5-androstene-17-one-3β-acetate, and Zn reduction of the 6β,19-oxide (1,2) (fig 2). Compound 2 was obtained by acetylation of compound 1 to 3β,19-diacetate, followed by selective hydrolysis of the 3β-acetoxy group by $KHCO_3$ in methanol (3,4), or, alternatively, both compounds 3 and 2 were obtained by selective acetylation of 3β,19-dihydroxy-5-androstene-17-one (compound 1). Since the latter substance was generously supplied by Dr. Ralph I. Dorfman, Syntex Corporation, it appeared this approach might be the most useful to explore.

The secondary acetoxy group at the C-3 position is the more easily removed by mild conditions attendant in the HCO_3-methanol reagent, suggesting that the 19-hydroxy group is the most difficult to acetylate, steric hindrance being the overriding deterrent in both instances.

Based on preliminary work, the selective acetylation of 45 g of 3β,19-hydroxy-5-androstene-17-one was carried out in benzene-pyridine with 1 mol equivalent of acetic anhydride. The reaction sequence was monitored by silica gel thin-layer chromatography (benzene/ethanol, 85/15). After 27 hours of reaction, most of the dihydroxy compound had disappeared, and two principal products and a minor product were apparent from the monitoring process. The reaction was terminated, and the mixture was resolved by tubular nylon column chromatography on silica gel (8 × 80 cm, 4 kg silica

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[2] Department of Urology, Indiana University School of Medicine, Indiana University-Purdue University, Indianapolis.

[3] *Reprint requests to:* Harry E. Hadd, PhD, Department of Urology, Indiana University School of Medicine, Indiana University-Purdue University, Indianapolis, Ind 46202.

gel). Development was accomplished with 7 lbs of diethyl ether; puncture sampling technique revealed the separation of the four substances (5,6). The most prominent product was identified as 19-acetoxy-3β-hydroxy-5-androstene-17-one. It was isolated by elution of the proper segment of the nylon column with methanol (weight, 16.8 g; yield, 23.6%) and showed a single spot by silica gel thin-layer chromatography (benzene/ethanol, 85/15). The melting point was 126.5°–127.5° C (123°–124° C [*see* ref 3], 121°–123° C [*see* ref 7]). The physical data by nuclear magnetic resonance (courtesy of Dr. J. Grutzner, Department of Chemistry, Purdue University) were as follows (in ppm): $18CH_3$, 0.92; CH_3CO, 2.09; 3OH, 2.97; 3H (broad), 3.40–3.60; $19CH_2$ quartet, 3.88, 4.00, 4.66, 4.57; 5-vinylic, 5.63. Mass spectra (courtesy of Dr. Graham Cooks, Department of Chemistry, Purdue University) were as follows: small M+, 346; principal ion, 328; 346–$18(H_2O)$ = 328; 328– $60(CH_3CO_2H)$ = 268; 346– 60 (CH_3CO_2H) = 286. Principal infrared spectra absorption bands: –OH (broad), 3500 cm^{-1}; C–H stretching, 2950 cm^{-1}; C = O, 1735; sh, 1745 cm^{-1}; acetyl, 1250 cm^{-1}.

The second most prominent product weighed 11.3 g and was identified as 3β,19-diacetoxy-5-androstene-17-one (melting point, 105° C (103°–105° C [*see* ref 8]). The physical data by nuclear magnetic resonance were as follows: CH_3, 0.94; CH_3CO, 2.06 and 2.10; H–4,s, 2.37 and 2.45; $19CH_2$ quartet, 3.90, 4.02, 4.50, 4.63; 3H (broad), 4.40–4.48; 5-vinylic, 5.69 (broad, 5.60–5.74). Mass spectra were as follows: no visible M+; 388– $60(CH_3CO_2H)$ = 328; 328–$43(CH_3CO)$ = 295; 328– $73(CH_3CO \cdot OCH_2)$ = 255; 328– $60(CH_3CO_2H)$ = 268; 286– $18(H_2O)$ = 268.

The third compound weighed 3.1 g and has not been further characterized. It probably represents the 3β-monoacetate. It was intermediate in polarity (by thin-layer chromatography) between the 19-monoacetate and the 3β,19-diacetoxy compounds.

The relatively greater yield (five times more) of the 19-acetoxy compound than of the somewhat less polar 3β-acetoxy compound indicated that monoacetylation of 3β,19-dihydroxy-5-androstene-17-one was a feasible method of selectively covering one hydroxyl group of this available starting material. Turner et al (4) obtained the 19-acetoxy compound in 70% yield by limited deacetylation of 3β,19-diacetate (3β-monoacetate being a minor product).

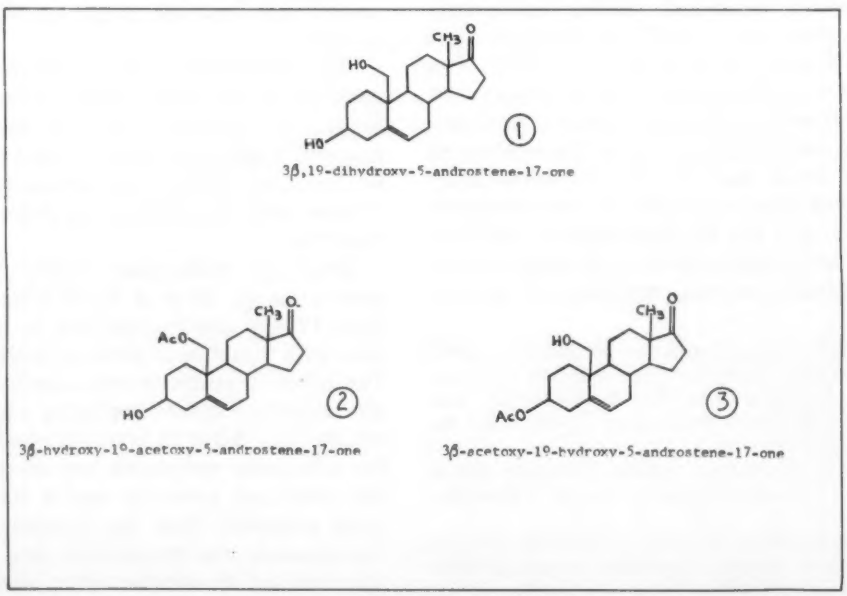

FIGURE 1

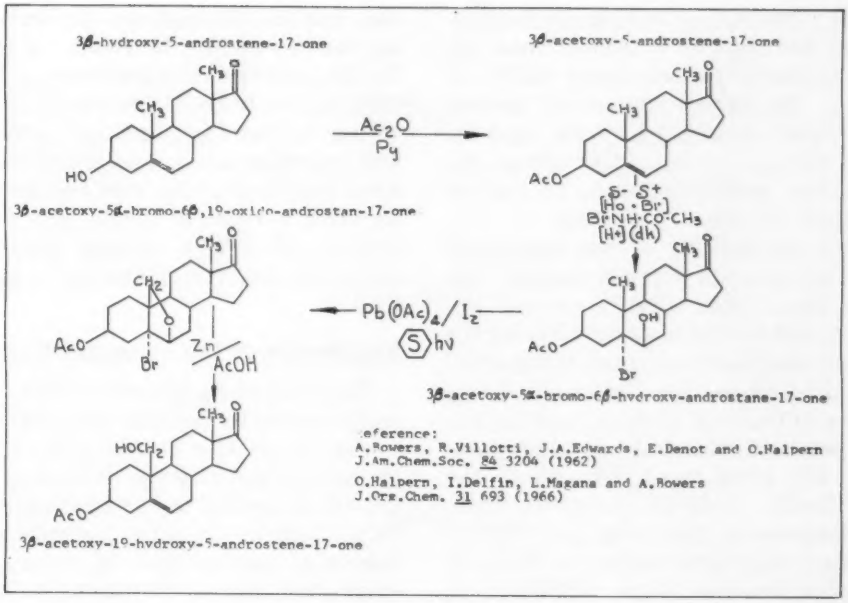

FIGURE 2

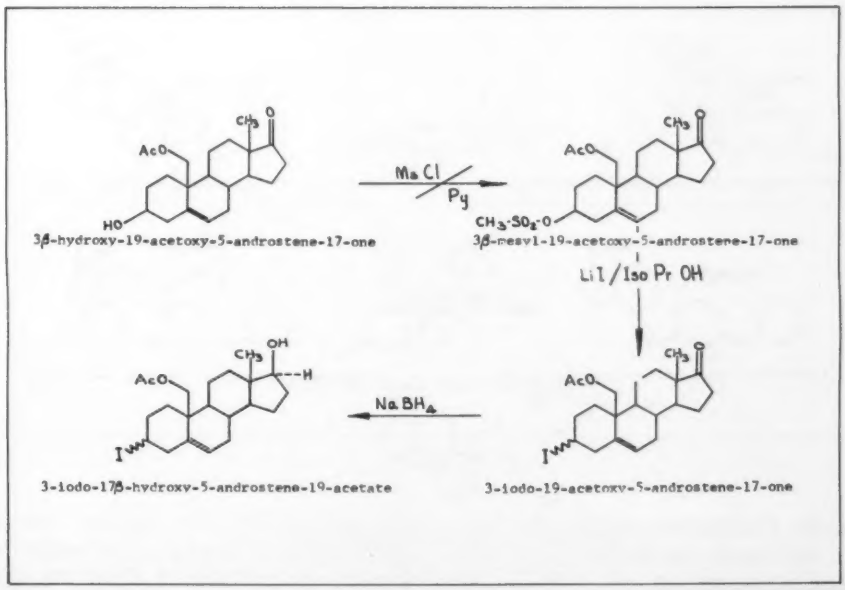

FIGURE 3

Employing 19-acetoxy-3β-hydroxy-5-androstene-17-one, two reaction sequences were followed for insertion of the iodo group (fig 3). In this sequence, the stereoisomerism of position C-3 was probably diminished by the repeated exposure of the C_3-I to LiI. The multiple inversion reaction probably results in loss of stereospecificity at the C-3 position.

The second sequence (fig 4) was directed at placing the iodo group at the C-19 position. The tetrahydropyranyl ether (THP) covered the hydroxyl group at the C-3 position, allowing the stronger basic conditions to hydrolyze the acetyl group from the C-19 position. This was followed by mesylation of the C-19 hydroxyl, and the displacement reaction with LiI in isopropanol (refluxing) for 2 hours brought about the insertion of 19-$CH_2 \cdot I$ (fig 5). During the work-up, tetrahydropyranyl ether was lost. The infrared spectra were quite similar to those of 3β-hydroxy-5-androstene-17-one (DHEA), ie, OH stretching at 3500 cm^{-1}. Tables 1–3 show the physical constants, the melting points, the nuclear magnetic resonance data of the 19CH_2 quartet, and the data on the silica gel plates for each compound.

Preliminary biologic testing of the first iodo compound, 3-iodo-19-acetoxy-5-androstene-17-one, and its 17β-hydroxy derivative has been carried out by Mr. M. Hanlin of the Eli Lilly Co. The activities of these compounds have been compared to those of 3β-hydroxy-5-androstene-17-one in the castrated rat prostate cytosol DHT-receptor assay. The preliminary observations suggest that the iodo and acetoxy groups on DHEA retained about 15%–25% of the activity of DHEA toward diminishing the ability of the cytosol receptor system to bind 5α-DHT.

Significance of This Projected Work

The areas of significance of this work are directly related to the early detection of prostatic cancer. In addition to this, there exists the potential of discerning the physiology of prostate growth as related to the hormonal continuum. Thus, benign prostatic hyperplasia may be detectable earlier than is currently possible using the digital "visualization" procedure. This circumstance may permit earlier control, perhaps via medical control, through an anti-androgen (nonhormonal agent). Such substances that interfere with the binding of the 5α-DHT at the prostate chromatin-binding site could be developed and used in treatment.

As we view this work, the critical questions

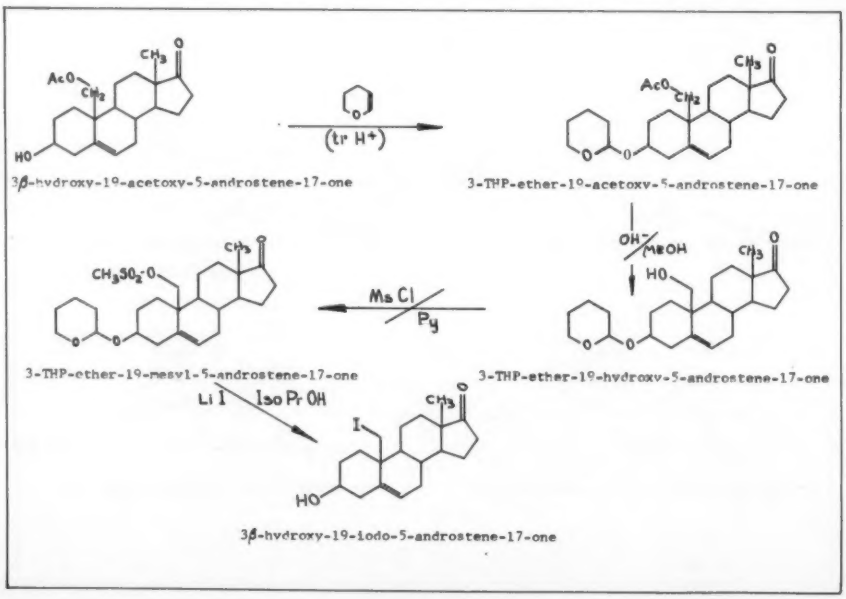

FIGURE 4

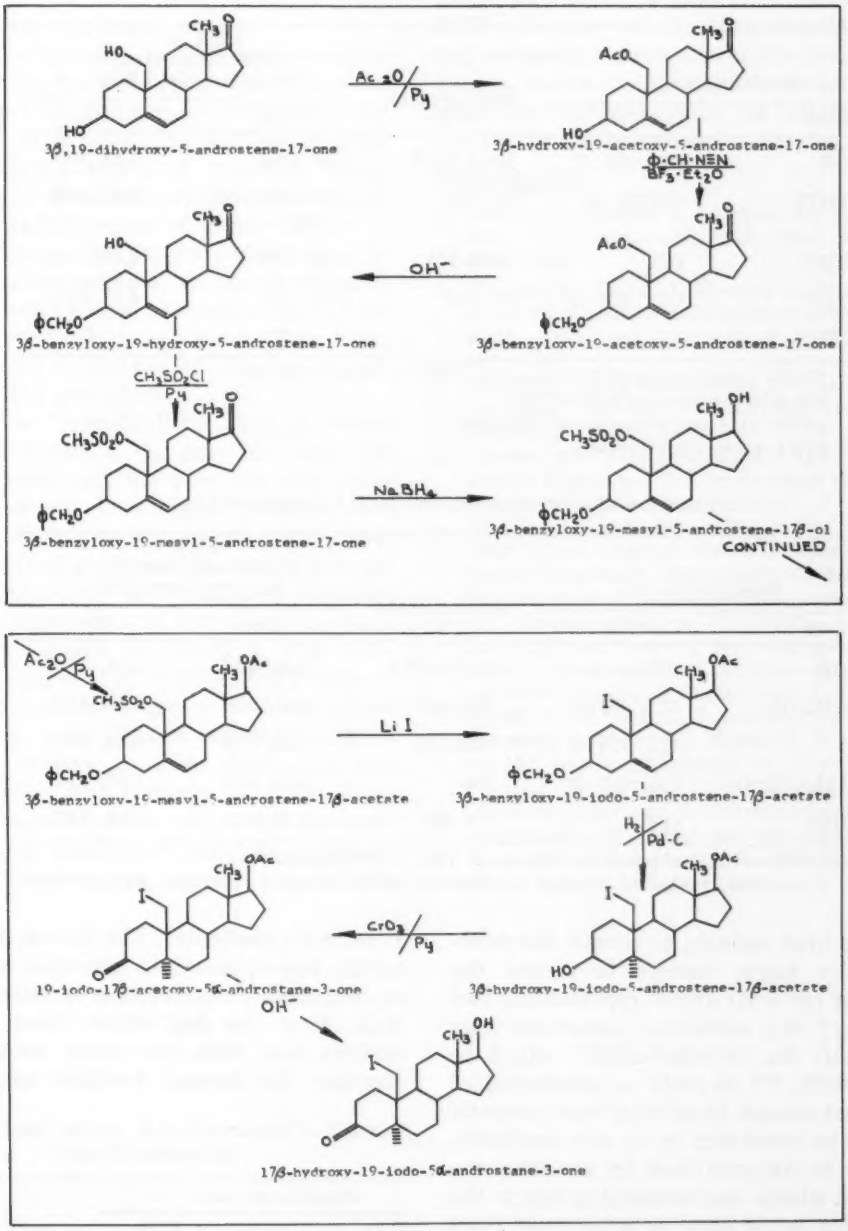

FIGURE 5

are as follows: (*a*) can the 17β-hydroxy-19-iodo-5α-androstan-3-one indeed be synthesized by the route set forth, or modified, to yield a substance that will be stable for radioiodination? (*b*) Will the isotopically labeled steroid be abstracted selectively from the route of administration by the prostate and will it be retained long enough, and with ratio to non-

TABLE 1.—Physical constants (nucleus: 5-androstene-17-one)

Substituents at—		Melting point (°C)	NMR of C-19CH_3 quartet* (in ppm from TMS)	
C-3β	C-19CH_3		19a	19b
OH	$CH_3 \cdot CO \cdot O$	126.5–127.5	4.46, 4.58	3.88, 4.00
THP§	$CH_3CO \cdot O$	117–119	4.44, 4.48, 4.56, 4.60	3.90, 4.02
THP	OH	165.5–168	3.89, 3.99	3.40, 3.75†
THP	$CH_3 \cdot SO_2 \cdot O$	114–117	4.47, 4.67	4.17, 4.27
OH	I	63–65	‡	‡

*NMR = nuclear magnetic resonance; TMS = trimethylsilyl.
†Doublet obscured by H-6' of THP.
‡NMR data are incomplete at this time.
§THP = tetrahydropyranyl.

TABLE 2.—Physical constants (nucleus: 5-androstene-17-one)

Substituents at—		Melting point (°C)	NMR of C-19CH_3 quartet* (in ppm from TMS)	
C-3β	C-19CH_3		19a	19b
OH	$CH_3CO \cdot O$	126.5–127.5	4.46, 4.58	3.88, 4.00
CH_3SO_2O	$CH_3 \cdot CO \cdot O$	138–141	4.45, 4.57	3.88, 4.00
I	$CH_3 \cdot CO \cdot O$	143–146	4.48, 4.60	3.89, 4.01
$CH_3 \cdot CO \cdot O$	$CH_3 \cdot CO \cdot O$	105	4.51, 4.63	3.90, 4.02
OH†	OH	219–221	3.65, 3.75	3.36, 3.46

*NMR = nuclear magnetic resonance; TMS = trimethylsilyl.
†Compound furnished through the courtesy of Dr. Ralph I. Dorfman, Syntex Corp.

target areas high enough, to permit the selectivity of the Anger camera to record the outlined "hot (or cold) areas" representing foci of pathology? One additional possibility may present itself: the 19-iodo-5α-DHT might be exchanged with ^{131}I to yield a radioiodinated product great enough in activity that prostatic cancer may be irradiated by in situ perfusion. This might be accomplished by encasing the compound in silastic and embedding this in the prostate. This would serve as a reservoir from which the compound would be under constant release.

In modern clinical and investigative urology the hormonal dynamics of the normal prostate, benign prostatic hyperplasia, and prostatic carcinoma are largely unknown (and nearly unmeasurable routinely). The disease processes of benign hyperplasia and prostatic cancer have no comparable counterpoint in laboratory animals. Even the dog, which more closely resembles man than any other animal, cannot simulate the human prostate satisfactorily;

TABLE 3.—Comparison of R_f values (basic nucleus: 5-androstene-17-one)*

Substituents at—		R_f value
C-3β	C-19	
OH	Acetoxy	0.43
THP	Acetoxy	0.79
THP	OH	0.56

*Silica gel thin-layer chromatography (benzene/ethanol, 9/1).

therefore, it is absolutely essential to study the disease processes in man.

Prostatic malignancy is the most common cancer in men in this country, and it is the third leading cause of death from cancer in men over the age of 55. With advancing numbers of older men, increasing numbers of prostatic cancer patients will be seen, and prostatic cancer will become the leading cause of cancer deaths in men over the age of 75. In addition to the numbers of older patients, an increasing incidence of prostatic cancer has been seen over the past decade, particularly in the American black male, in whom it has nearly doubled in frequency over the past decade.

Staging, the "visualization" through rectal digital examination, is the only technique for clinically determining the prostate size, aside from urethroscopy. It is highly probable that about a 1-cm nodule on the posterior surface of the hypertrophied gland represents the average size nodule that can be recognized digitally. Already this represents, at the minimum, stage B, and there might be about a 15%–20% overlap into stages C or D. Since the combined surgical cure rate for stages A and B is about 80% (for 5- and 10-year survivals), it is essential to achieve accurate staging in this disease. A treatment protocol based on more accurate clinical staging will select patients properly, those considered curative by means of surgical intervention. By the same token, prostatic tissue incorporation of 19-iodo-5α-DHT will allow specific bone scan data regarding metastatic prostatic carcinoma. Stage D lesions would be diagnosed earlier and unnecessary surgery, with its attendant poor results, would be avoided.

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Specificity of Cell Membrane Antigens in Prostatic Cancer[1]

George E. Brannen,[2,3] Diana M. Gomolka,[2] and Donald S. Coffey[4,5]

SUMMARY

Mice bearing a 3-methylcholanthrene-induced fibrosarcoma (MCAM-7) transplant in the right leg underwent surgical excision of the tumor and showed specific resistance to subsequent challenges with that identical tumor line. An in vivo response to tumor-specific antigens (MCAM-7 antigen) solubilized by hypertonic potassium chloride was measured by 24-hour footpad swelling response in mice immunized to the tumor from which the antigens were extracted. These observations suggested that the transplantable MCAM-7 fibrosarcoma could produce immunity toward the solubilized MCAM-7 tumor antigens and that this tumor immunity could be measured by footpad swelling response to injection of the solubilized antigens, an indication of cell-mediated immunity. The footpad swelling response was also monitored in relation to the extent of tumor growth. Mice received MCAM-7 tumor transplants by injection of 5×10^6 tumor cells and were tested for footpad swelling response at intervals following tumor transfer. A significant footpad response to injected MCAM-7 antigens was present 10 days following tumor transfer; at this time signs of tumor growth were only minimally detectable. The footpad swelling response to injected antigens disappeared by 28 days following initial tumor transfer; at this time the tumor diameters were in excess of 1.0 cm. Surgical removal of tumor at this point promptly restored footpad responses within 24 hours. Similar techniques have been applied to patients bearing adenocarcinoma of the prostate, where skin testing was substituted for the measurement of footpad swelling in animals. Seven patients with known prostatic carcinoma were given intradermal injections of soluble tumor antigens extracted from their own tumors. Three of the seven patients exhibited a cutaneous delayed type hypersensitivity response to the injected autologous tumor extracts. No positive reactions were observed in response to solubilized components of control tissues, including benign prostatic hyperplasia. The significance of the demonstrated concomitant immunity in these patients has not been resolved. However, these observations suggest that some patients bearing adenocarcinoma of the prostate can exhibit an immunologic response to specific antigens present in their own neoplasms.

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[2] The James Buchanan Brady Urological Institute, The Johns Hopkins Hospital, Baltimore, Md.

[3] Dr. Brannen is an American Cancer Society Fellow.

[4] Department of Pharmacology, The Johns Hopkins University School of Medicine, Baltimore, Md.

[5] *Reprint requests to*: George E. Brannen, MD, The James Buchanan Brady Urological Institute, The Johns Hopkins Hospital, Baltimore, Md 21205.

ANIMAL EXPERIMENTS

Since the pioneer tumor transplantation experiments of Gross (1), Foley (2), and Prehn et al (3), evidence has continued to accumulate suggesting that some animal tumors may bear antigens distinct from antigens of other host tissues. In addition, these tumor antigens seem to have immunogenic potentials, eliciting a weak tumor-specific immune response in the tumor-bearing host. When using carcinogen-induced animal tumors, the immune response to tumor antigens is frequently a cell-mediated (delayed type hypersensitivity) response and is generally specific for the immunizing tumor cell line.

Several methods of preparing soluble tumor-specific antigens (TSA) from chemically induced rodent tumors have been reported. The methods have included manual homogenization plus freeze-thaw (4), saline extraction followed by ultracentrifugation (5), proteolytic digestion of insoluble membrane fractions (6), sonication, and hypertonic (3 M) potassium chloride extraction (7). The various antigen preparations were shown to be immunologically active and tumor specific by production of transplantation immunity to the extracted tumor line (8–10), by elicitation of typical delayed type hypersensitivity skin responses in animals immunized to the tumor line extracted (7,9), or by inhibition of macrophage migration when incubated with peritoneal exudate of animals immunized to the tumor extracted (9,11).

The purpose of the present study was to demonstrate the utility of soluble TSA of 3-methylcholanthrene (MCA)-induced murine fibrosarcomas as footpad test antigens for monitoring immunologic reactions to syngeneic tumors. Studies were performed to measure changes in footpad responses to soluble TSA (prepared by the hypertonic potassium chloride method) as influenced by progressive tumor growth and tumor excision.

MATERIALS AND METHODS

Animals

Male (BALB/c × DBA_2)F_1 hybrid mice, 9–11 weeks of age, obtained from Cumberland View Farms, Oak Ridge, Tennessee, were used in these studies. These hybrid mice are referred to as $CD2F_1$.

Tumor Cell Lines

Fibrosarcomas were induced in nine male $CD2F_1$ mice by subcutaneous (sc) injection of 2 mg of MCA in 0.10 ml of trioctanoin. Palpable tumors arose approximately 3–4 months later. These MCA-induced murine fibrosarcomas were coded in order of appearance (ie, MCAM-1, MCAM-2, ... MCAM-9). After excision, a cellular suspension of each tumor was made by homogenization in phosphate-buffered saline (PBS, 0.1 M, pH 7.4) with the aid of a Ten Broeck glass homogenizer. Cells were washed once and resuspended in PBS for transfer of tumor. Tumor transfer was carried out by sc injection of 10^7 viable tumor cells (trypan-blue exclusion). The transfer generation of each tumor was monitored, G_1 being the first passage generation, G_2 the second, etc. Studies were performed using G_2, G_3, and G_4 generations.

Immunization to Tumors and Proof of Transplantation Immunity

Immunization to the tumors was performed using techniques similar to those of Prehn and Main (3). Tumor cell suspensions containing 5×10^6 cells in 0.05 ml of PBS were injected into the right footpads of $CD2F_1$ mice. When tumors reached 0.5–1.0 cm in greatest diameter, the tumors were excised by right leg amputation under ether anesthesia. Amputation of the right leg was similarly performed in control mice. One week after amputation the experimental and control mice were challenged in the remaining footpads with graded doses of tumor cells to test for transplantation immunity. Mice that resisted tumor growth were considered immune if the same tumor cell dose produced growth in control mice. Immune animals that were to be footpad tested were given a "booster" injection of 10^5 viable tumor cells in the remaining footpad 7 days before testing.

Preparation of Soluble TSA

Solubilization of TSA by hypertonic (3 M) potassium chloride was used in these studies. The procedure was carried out using the method of Meltzer et al (7). Instead of ul-

trafiltration, the supernatants containing extracted TSA were lyophilized and the powder was reconstituted to one tenth of its original volume. Protein concentrations of the soluble extracts were determined by the biuret method of Bernhard and Scher (12). Protein concentrations of TSA extracts were adjusted to a concentration of 7.5 mg/ml for use in footpad testing. Bacterial cultures of the extracts were negative prior to use in footpad testing. Since our footpad tests with this material elicited little nonspecific response, further purification procedures suggested by Meltzer et al (7) were not carried out.

Method of Footpad Testing

Evidence that measurement of footpad swelling in the mouse and rat could be used to detect specific cell-mediated immunity to sheep erythrocytes was presented by Nelson and Mildenhall (13) and Parish (14) respectively. Successful repetition of similar experiments in male $CD2F_1$ mice in our laboratory established the basis for use of the footpad swelling response in our experiments.

In our studies 0.05 ml of TSA preparation was injected sc into the appropriate footpad. In addition to baseline measurements, the footpad thicknesses were measured 24 hours after injection, using a dial caliper gauge A02T (Schnelltaster, H.C., Kroplin GmbH, Schlüchtern, Hessen, Germany) (14). The footpad thicknesses were determined by the average of five independent measurements. The millimeters of footpad swelling (footpad thickness at 24 hours minus baseline thickness) for each group of mice were averaged. Animal groups were coded, and measurements were made in a blinded manner.

Representative biopsies of responding and control footpads were taken for histologic examination. The readings of responding footpads were consistent with classic criteria for delayed type hypersensitivity response. Control tumor antigens elicited no footpad swelling response and elicited no response of cellular infiltration as determined by histologic examination.

Statistical Analysis

The data of footpad swelling response were analyzed by the multiple-range test of Duncan (15), referring to the revised critical values for this test (16). Significance was accepted at the 5% level, but analysis at the 1% level was also performed.

RESULTS

Relative Immunogenicity of MCA-Induced Tumors

The MCA-induced fibrosarcomas were tested individually for immunogenicity in $CD2F_1$ male mice. Groups of 30 mice were immunized to each of six different MCA tumors by excision of the tumor as outlined above. On the same day the right legs of 30 control mice for each of the six tumors were also amputated. One week after amputation the experimental and control mice were challenged in the remaining footpads in groups of ten with 10^4, 10^5, and 10^6 tumor cells (viability 20%, trypan-blue exclusion) in 0.05 ml of PBS. Individual mice were examined twice weekly for 4 months for the presence of tumor growth in the challenged footpad. The results are summarized in table 1. Four of the tumors (MCAM-3, MCAM-4, MCAM-7, and MCAM-8) were able to elicit transplantation immunity (ie, they demonstrated immunogenicity) with the method employed. The most immunogenic MCA tumor, MCAM-7, was chosen for our remaining studies. Additional studies indicated that the minimal dose of MCAM-7 tumor cells required for growth in 80%–100% of mice was 5×10^3 cells.

The specificity of transplantation immunity to MCAM-7 was confirmed by challenging MCAM-7-immune mice with MCAM-8. The results are summarized in table 2. In subsequent experiments MCAM-8 was used as a tumor specificity control tissue.

The results of studies designed to measure changes in the ability of a given transplant generation of MCAM-7 to produce transplantation immunity to the same tumor generation are noted in table 3. The decline in transplantation resistance in mice immunized to G_2 versus G_5 and G_6 may reflect changes in immunogenicity and/or resistance of the tumor cells to immune destruction. As noted above, on the basis of this study we confined our studies to tumors of the first four transplant generations.

TABLE 1.—Relative immunogenicity of 6 fibrosarcomas induced by MCA in male $CD2F_1$ mice

	No. of tumor cells injected for challenge*					
	10^4		10^5		10^6	
Tumor	Control	Tumor excised	Control	Tumor excised	Control	Tumor excised
MCAM-1	0/5†	0/10	0/5	1/10	2/5	4/10
MCAM-2	0/5	1/10	4/5	7/10	5/5	9/10
MCAM-3	0/10	0/5	4/10	0/5	3/10	0/6
MCAM-4	0/5	0/2	3/5	1/4	5/5	2/3
MCAM-7	7/10	0/10	10/10	0/10	8/10	0/10
MCAM-8	0/5	0/5	4/5	0/5	5/5	5/5

*G_1 tumor was grown in the right legs of 10 mice for each group. The right legs were amputated. The left legs were challenged with G_1, 7 days after tumor excision.
†No. of animals developing tumor/total No. challenged.

TABLE 2.—Specificity of transplantation immunity to MCAM-7

	No. of mice developing tumor/total No. challenged	
No. of MCAM-8 challenging cells injected*	Control mice	Mice immune to MCAM-7
10^4	0/6	0/6
10^5	2/6	3/6
10^6	5/5	4/4

*Mice immune to MCAM-7 were "boosted" with 10^5 MCAM-7 cells 7 days before challenge with MCAM-8 cells.

TABLE 3.—Changes in ability of varying transplant generations of MCAM-7 to produce transplantation immunity to 10^5 tumor cells of the immunizing transplant generation

Transplant generation of MCAM-7	Fraction of mice resisting challenge of 10^5 MCAM-7 tumor cells*
G_2	30/30†
G_3	67/69
G_4	32/36
G_5	28/38
G_6	46/79

*Mice were challenged in the remaining footpad with 10^5 MCAM-7 tumor cells, arising from the same transplant generation as the MCAM-7 tumor which was excised 7 days before challenge.
†Transfer of 10^5 MCAM-7 tumor cells into control animals resulted in tumor growth in 100% of animals for each transplant generation.

Footpad Swelling Responses to Solubilized Tumor Antigens

Seven days after booster challenge (10^5 MCAM-7 tumor cells in 0.05 ml of PBS), MCAM-7-immune mice and control mice were given footpad injections of solubilized MCAM-7 or MCAM-8 tumor antigens in groups of ten. The footpad swelling responses 24 hours after injection of tumor antigens are represented in figure 1. Only MCAM-7-immune mice showed a significant footpad swelling response to solubilized MCAM-7 tumor antigens. No swelling response to MCAM-8 tumor antigens was noted after footpad injections of MCAM-7-immune or control mice.

Variation of Tumor-Specific Footpad Swelling Responses With Tumor Growth and Excision

Groups of 50 male $CD2F_1$ mice were injected with 5×10^6 cells of MCAM-7 or MCAM-8 tumor in the right footpads. Seven, 10, 19, and 28 days after tumor injection, five mice from each group were tested in the left footpads by injection of hypertonic potassium chloride tumor antigen extract of MCAM-7 or MCAM-8. The largest diameter of the tumor in the right leg was recorded in parallel with administration of footpad injections. Measurements of footpad swelling responses 24 hours after tumor antigen injections are presented graphically in figure 2. Significant swelling responses specific to MCAM-7 tumor antigens

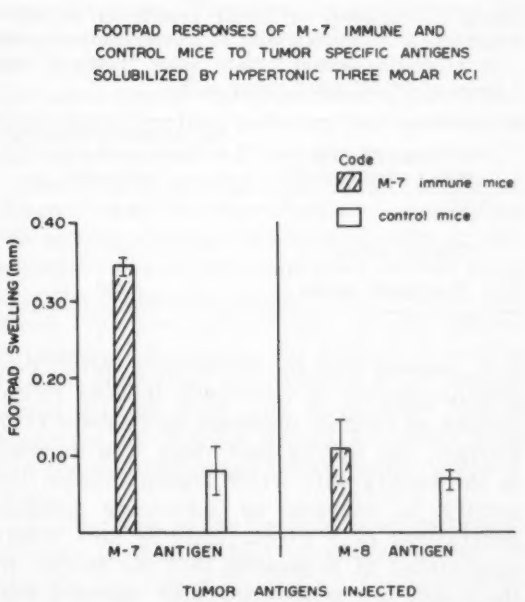

FIGURE 1.—Footpad responses of MCAM-7-immune and control mice to TSA solubilized by hypertonic 3 M potassium chloride.

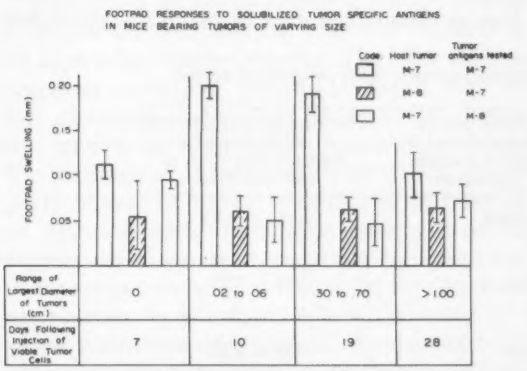

FIGURE 2.—Footpad responses to solubilized TSA in mice bearing tumors of varying sizes.

were detectable 10 and 19 days after injection of MCAM-7 (but not MCAM-8) tumor cells. Twenty-eight days after tumor injections no specific footpad responses could be elicited by MCAM-7 antigens. At this time, tumor size was greater than 1.0 cm in largest diameter.

Groups of five male $CD2F_1$ mice were injected with 10^7 MCAM-7 cells in the right footpads. When tumor size exceeded 1.0 cm in largest diameter, the mice were tested in the left footpad by injection of the hypertonic potassium chloride extract of MCAM-7. No footpad responses to extracted tumor antigens could be elicited in mice bearing these large tumors. The MCAM-7 tumors were completely excised, and the mice were tested in the remaining footpads with TSA extracts of MCAM-7 and MCAM-8 in groups of five at 1, 3, 5, 7, 21, 32, and 56 days after tumor excision. Measurements of 24-hour footpad responses are displayed in figure 3. The data clearly indicate that a specific 24-hour footpad response to hypertonic potassium chloride extracts of the excised MCAM-7 fibrosarcoma returned within 1 day after tumor excision. The 24-hour footpad responses specific to the solubilized MCAM-7 TSA persisted through Day 32 but subsided prior to Day 56 after MCAM-7 tumor excision.

Abrogation of Tumor-Specific Footpad Response With Pooled Progressor Serum

Progressor serum was defined as serum collected from $CD2F_1$ male mice bearing MCAM-7 tumor with diameters greater than 1.0 cm and showing no 24-hour specific footpad response to a hypertonic (3 M) potassium chloride extract of the MCAM-7 tumor (protein concentration, 7.5 mg/ml). Twenty mice immunized to MCAM-7 fibrosarcoma showed a positive footpad response to soluble MCAM-7 TSA. Fourteen days after the initial footpad test the mice underwent footpad testing with MCAM-7 or MCAM-8 TSA in groups of five.

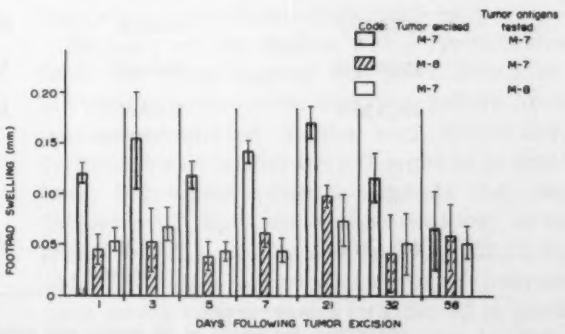

FIGURE 3.—Measurements of 24-hr footpad responses.

Each tumor antigen extract was tested in mice given either 0.60 ml of pooled progressor serum or 0.60 ml of pooled control serum intravenously (iv) 1 hour before footpad injection of the TSA. Measurements of footpad swelling responses to TSA in MCAM-7-immune mice after injection of sera are displayed in table 4. Although no significant abrogation of footpad response to soluble MCAM-7 TSA by progressor serum was measured for the experimental group as a whole, it appears that three individual MCAM-7-immune mice may have had their 24-hour footpad responses abrogated by progressor serum.

In another attempt to demonstrate "serum blocking activity," 26 male $CD2F_1$ mice immunized to MCAM-7 were given 0.60 ml of pooled sera iv 7 days after a "booster" challenge of 10^5 MCAM-7 tumor cells in the remaining footpads. Incidence of tumor growth in the groups of MCAM-7 immunized mice given no serum, control serum, or progressor serum is shown in table 5.

TABLE 5.—Incidence of tumor growth in MCAM-7-immune mice injected with no serum, control serum, or progressor serum 7 days after "booster" challenge of 10^5 MCAM-7 tumor cells

Type of pooled serum administered	No. of mice developing tumor/total No. challenged
None	2/9
Control serum	3/7
Progressor serum	7/10

DISCUSSION

The present observation that four of six MCA-induced fibrosarcomas were immunogenic is in keeping with the well-known variation in immunogenicity of chemically induced rodent tumors, as recently discussed by Baldwin (17). Further, the finding that there is a decrease in the facility with which transplantation immunity is induced by advancing passage generations of a given tumor is also widely appreciated. It is possible that the surface of the tumor cell is modified with repeated passage to render it less immunogenic and/or more resistant to immunologically specific mediated injury. Such changes may occur because of immunoselective pressures similar to those suggested by Prehn (18) to explain the variation in immunogenicity in primary carcinogen-

TABLE 4.—Abrogation of tumor-specific footpad response with progressor serum

Source of soluble TSA	Test animals*	Pooled serum administered iv before footpad injection	Footpad swelling in mm ± SE 24 hrs after footpad injection of soluble TSA
MCAM-7	MCAM-7 immune	Control	0.14 ± 0.03
MCAM-8	MCAM-7 immune	Control	0.07 ± 0.01
MCAM-7	Control	None	0.03 ± 0.02
MCAM-8	Control	None	0.05 ± 0.01
MCAM-7	MCAM-7 immune	Progressor	*a.* 0.14† *b.* 0.11 *c.* 0.07 *d.* 0.06 *e.* 0.03
MCAM-8	MCAM-7 immune	Progressor	0.04 ± 0.02

*Five mice per group.

†Footpad responses at 24 hours are noted for individual mice in group tested for serum abrogation of tumor-specific response.

induced tumors. Whatever the mechanisms involved, the present studies further underscore the practical point that investigations involving tumor-specific transplantation antigens and transplantation immunity are best confined to the first four or five transplant generations.

The present studies indicate the ability of the swelling of the mouse footpad in monitoring the specific cellular (24-hour delayed hypersensitivity) immunity employing soluble preparations containing active TSA. Halliday and Webb (19) were able to show specific delayed hypersensitivity using the footpad technique in mice immunized to syngeneic MCA tumors. These authors were able to measure specific responses in immune mice with living cells, but not with frozen and thawed tumor preparations.

The decision to use the hypertonic potassium chloride method of TSA solubilization for our studies was based in part on the arguments as to its advantages over other methods in obtaining soluble transplantation antigens (20), its use for obtaining soluble TSA in animals (7) and man (21), and its simplicity and reproducibility.

Mice given an initial tumor inoculum showed a specific 24-hour footpad response as early as 10 days after tumor transfer. The TSA footpad response remained positive through Day 19 but disappeared by Day 28 when the tumor had grown to a diameter exceeding 1.0 cm. The findings of tumor-specific immunity in the early phases of tumor growth and the decline of tumor-specific immunity with increasing tumor size have been reported by a number of investigators using a variety of experimental approaches. In situations where challenges of a given tumor are administered at various times after an initial transfer of the tumor, animals in general have been shown to specifically resist second challenges (concomitant immunity) until a certain critical size is reached. Challenges given when this critical size is reached and afterwards are not resisted (22–25) unless the primary tumor is first removed (26). The mechanism by which concomitant immunity declines or is lost is not entirely clear.

Several authors have also shown that lymphoid or peritoneal exudate cells from animals bearing small tumors are able to adaptively transfer immunity to that tumor but are unable to do so when the tumor is large (22,26–30). In addition to animal studies, Diehl et al (31) observed that lymphocytes from the peripheral blood of patients with localized nephroblastoma were inhibitory to the growth of tumor cells while lymphocytes obtained from patients with metastatic tumor were inactive.

In the present studies, animals with large tumors and negative to footpad testing with TSA developed positive delayed hypersensitivity to TSA within 1 day after tumor excision. This is qualitatively consistent with data reported by other investigators. The ability to produce transplantation immunity to chemically induced or virus-induced tumors by tumor excision is well documented. In experiments with virus-induced mammary tumors (32) or tumors induced by Rauscher leukemia virus (33, 34), surgical removal was performed when tumors were large and when it could no longer be demonstrated that peritoneal cells were reactive to tumor target cells. Reactive peritoneal cells appeared 12–23 days after excision. Belchradek et al (22) studied a similar situation employing a carcinogen-induced tumor. These authors noted that measurable reactivity of peritoneal cells did not return until 62 days after resection of a large tumor. In parallel experiments these same authors were able to show that resistance to challenge with appropriate tumor cells returned much earlier indicating a more sensitive assay of tumor transplantation immunity. The footpad response to TSA injection appears to be more rapidly indicative of restoration of immunity than other methods currently being used.

In many of the studies where lymphocytes from the tumor-bearing host have shown reactivity to tumor cells, blocking factors have been demonstrated in the sera (30,35–39). Initially, these factors were thought to be antibody, but recent evidence suggests that the factors may represent antigen-antibody complexes (40,41) and/or excess TSA (22,25,42, 43). The latter observations are of interest since excess antigen would be expected to block effector lymphocytes as demonstrated by Ash-

erson et al (43). Failure to find sensitized lymphocytes in tumor-bearing hosts, therefore, might represent a failure to adequately "wash off" antigen from lymphocytes used in the various in vitro assays. This notion is supported by the observation of Belchradek et al (22) who observed that peritoneal exudate cells obtained from animals with large tumors were incapable of reactivity to these tumor cells in vitro. Yet these same workers noted that sera from tumor-bearing animals were capable of abrogating the specific cytotoxic effect of peritoneal cells obtained from immunized animals. Furthermore, this block acted on the effector cells rather than the tumor target. Most recently, Wright et al (44) noted that unreactive lymphocytes obtained from rats with Gross virus-induced leukemia became active in ^{51}Cr release tests for cell-mediated immunity when these cells were preincubated for 4–24 hours in vitro (shedding of antigen?).

The expression of cellular immunity to tumors as a positive footpad swelling response is certainly the result of an extremely complex series of reactions. The explanations for loss of cell-mediated immunity to TSA with large tumors and the return of cell-mediated response after tumor excision could be further resolved by correlations of in vitro assays with the in vivo responses observed. Studies of different facets of the cell-mediated response might relate the dynamics of in vivo TSA responsiveness to lymphocyte repression, serum blocking factors, or simply catabolic phenomena in the host.

HUMAN STUDIES

Since the techniques used in our animal tumor model suggested the presence of concomitant tumor immunity, we investigated cutaneous responses to autologous soluble tumor antigens in patients bearing adenocarcinoma of the prostate. The limited information related to immunologic studies of adenocarcinoma of the prostate results from lack of adequate animal models and from lack of reliable tissue culture techniques.

Alterations of blast transformation of lymphocytes taken from patients bearing adenocarcinoma of the prostate (45–48)[6] suggest that cell-mediated immunoresponsiveness may play a role in these host-tumor relationships. Although several authors have demonstrated circulating fetal antigens in patients bearing adenocarcinoma of the prostate (49–52), these carcinoembryonic antigens have a broad spectrum of specificity.

Serologic studies of antigenic factors in human prostate have included normal, hyperplastic, and malignant prostatic tissue (48,53–55). In these studies the antigenicity of the tumors was not monitored in the hosts bearing the tumors. Therefore, the significance of these serum factors is not clear.

The potential for a host immune response to alter the growth of adenocarcinoma of the prostate is suggested only by the rare reports of regression of metastases after cryosurgery of the malignant prostate (56–59). The reports of Tass et al (60,61), Stewart (62), and Hollinshead et al (63) on other tumors, in conjunction with the success of our animal experiments, inspired us to attempt to elicit cutaneous responses after intradermal injections of prostatic tumor antigens solubilized using hypertonic (3 M) potassium chloride. The solubilized prostatic tumor antigens were tested in patients bearing the tumor from which the antigens were solubilized.

MATERIALS AND METHODS

Neoplastic or benign hyperplastic prostatic tissue was obtained from patients by means of transurethral resection, using the cutting current and the Stern-McCarthy Resectoscope. The tissue was minced in Hank's balanced salt solution. The minced tissue was washed by centrifugation, and solubilization of TSA was carried out using hypertonic (3 M) potassium chloride as previously described (7). Protein concentrations of TSA extracts were adjusted to 7.5 mg/ml. Cultures of the solubilized material were negative for bacteria in all instances.

In addition, the cell membranes of whole blood were extracted to soluble form by the same method of hypertonic potassium chloride. The solubilized components of blood served as

[6] Catalona WJ. Unpublished data.

control tissue and were compared to the solubilized components of prostatic tissue taken from the same patient.

Cutaneous responses were tested by administering 0.10 ml of experimental or control solubilized antigens intradermally in the forearm. The sites of injection were inspected 24 and 48 hours after antigen administration for cutaneous reactivity. An area of at least 5 × 5 mm of palpable induration at both 24 and 48 hours was considered a positive delayed type hypersensitivity cutaneous response. Negative responses had no induration. In all cases where a positive response occurred histologic interpretations of skin biopsies were consistent with classic criteria for delayed type hypersensitivity. Biopsies of nonresponding sites of control tissue injection revealed an absence of cellular infiltrate.

All prostatic cancer patients were skin tested with extracts from autologous prostatic adenocarcinoma, autologous blood, allogeneic hyperplastic prostate, and allogeneic malignant prostate. Tissue controls including extracts of blood taken from allogeneic tissue donors were also tested. In some instances, patients bearing adenocarcinoma of the prostate and not showing a cutaneous response to injections of TSA were tested for immunocompetence by intradermal skin tests using 0.10 ml of six commonly encountered antigens. These antigens include: intermediate strength purified protein derivative, 0.1 μg or 5 tuberculin units/0.10 ml; mumps skin-test antigen; varidase (streptokinase-streptodornase mixture), 40 and 10 units respectively; *Candida albicans* extract, 1:100 dilution of full-strength antigen; Trichophyton gypsum extract, 1:30 dilution; and histoplasmin, 1:100 dilution. Patients with reactive immune systems generally have a delayed type hypersensitivity cutaneous response to at least two of these six antigens. Patients responding to none of these test antigens are considered anergic.

RESULTS

Seven patients bearing adenocarcinoma of the prostate, clinical stage C or D, were tested with extracts of autologous tumor and autologous blood. In addition, the tumor-bearing patients were tested with extracts of allogeneic neoplasm, allogeneic hyperplastic prostate, and control extracts of blood cells from the allogeneic tissue donors. The results of skin responses are summarized in table 6. Three of the seven prostatic carcinoma patients showed a delayed type hypersensitivity cutaneous response specific to the tumor antigens extracted from autologous neoplasm. In one instance a mild cutaneous response was observed to allogeneic tumor antigens solubilized from adenocarcinoma of the prostate obtained from another patient. In no instances did solubilized components of benign prostatic hyperplasia or of blood cells elicit a cutaneous response. Posi-

TABLE 6.—Skin-test responses to solubilized antigens from adenocarcinoma of the prostate*

Patient No.	Stage of prostatic adenocarcinoma	Cutaneous responses: mm of induration in response to—			Tumor-specific response		Anergy
		Autologous extract of prostatic carcinoma	Allogeneic extract of prostatic carcinoma	Allogeneic extract from benign prostatic hyperplasia	Autologous	Allogeneic	
1	D	8 × 8	0	0	+	–	
2	D	6 × 6	0	0	+	–	
3	C	7 × 7	6 × 7	0	+	+	
4	D	0	0	0	–	–	Yes
5	D	0	0	0	–	–	Yes
6	C	0	0	0	–	–	?
7	D	0	0	0	–	–	?

*No cutaneous responses were elicited by solubilized components of autologous or allogeneic blood.

tive responses were proven in all instances by histologic studies of skin biopsies of the responding site. The results of histologic examination were consistent with classic criteria for delayed type hypersensitivity. All solubilized TSA eliciting a cutaneous response did not elicit such a response in other patients of the same age group.

Two of the four patients not responding to their tumor antigen extracts were available for further testing and both were anergic with the six commonly encountered antigens previously mentioned. Three patients who underwent transurethral resection for benign prostatic hypertrophy did not show cutaneous responses to soluble components of autologous or allogeneic hyperplastic prostate or to soluble allogeneic antigens of adenocarcinoma of the prostate.

DISCUSSION

These preliminary studies in man suggest that neoplastic cells of adenocarcinoma of the prostate may in some instances bear TSA to which the host may respond. Using prostatic tumor antigens solubilized by means of hypertonic (3 M) potassium chloride, a cell-mediated response can be elicited in the tumor-bearing host by intradermal injection of the solubilized autologous tumor antigens. The cutaneous responses appear to be tumor specific. In one instance (patient No. 3) a tumor-specific cutaneous response was observed to allogeneic as well as autologous solubilized prostatic tumor antigens. These cutaneous responses to autologous TSA in patients bearing adenocarcinoma of the prostate probably represent the first in vivo demonstrations of tumor-specific cell-mediated immunity in hosts with prostatic adenocarcinoma.

Although concomitant tumor-specific immunity has been demonstrated using in vitro testing in patients bearing nephroblastoma (64) and neuroblastoma (65,66), in vivo cutaneous responsiveness offers a simple test with possible clinical application. Preliminary data have shown that cutaneous responses can occur to autologous tumor antigens in many patients with other types of carcinoma (61–63); however, most of these responses occurred after tumor excision.

These preliminary observations of tumor-specific cutaneous responses in patients bearing adenocarcinoma of the prostate may have potential for diagnostic or prognostic tests.

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Detection of Prostatic Cancer

John T. Grayhack [1]

Detection of prostatic carcinoma at an early stage has traditionally depended on recognition of an area of induration by rectal examination and demonstration of anatomic abnormality by histologic study. Suggestions that frequent rectal examination would discover the great majority of carcinomas of the prostate while they were still confined to the gland are based on the assumption of localized development and progression of malignancy that produces alterations recognizable by palpation. Whether or not this is truly the situation has not yet been established. There is, however, evidence from histologic studies of autopsy and clinical specimens that a significant number of carcinomas of the prostate apparently develop in multiple areas of the gland so that the concept of a single progressive local growth is open to question. Recently, the observation that, in the presence of histologically identifiable malignancy, the lactic dehydrogenase (LDH) isoenzyme pattern shows a diffuse change in the histologically benign tissue to the predominance of the LDH-V characteristic of malignancy adds further question to the concept of a progressive development of an isolated cellular alteration. Because of these considerations, we have sought to evaluate the possibility of identifying patients that have a high risk of malignancy by studying the LDH isoenzymes in expressed prostatic fluid. Four groups of patients were studied: normal men under 40 years of age (group A), men with a histologic diagnosis of benign prostatic hyperplasia (group B), men with a histologic diagnosis of carcinoma (group C), and men with a clinical diagnosis of benign prostatic hyperplasia (group D) (table 1). LDH-V/I ratios were calculated for each individual in each group. None of the men under 40 without evidence of infection in the prostatic fluid had an LDH-V/I ratio exceeding 3 (fig 1). Approximately 14% of the men with a clinical diagnosis (fig 2) and 12% with a histologic diagnosis (fig 3) of benign prostatic hyperplasia had LDH-V/I ratios exceeding 3. In contrast 80% of the men with a histologic diagnosis of carcinoma including two of three with stage A and five of six with stage B lesions had an LDH-V/I ratio exceeding 3 (fig 4). The presence of greater than 10 white blood cells/high-power field in the prostatic fluid and a history of recent prostatitis were also associated with an LDH-V/I ratio exceeding 3 in a high percentage of patients with clinically and histologically benign prostates. These findings support the concept that a diffuse cellular metabolic change preceding a histologic change is fre-

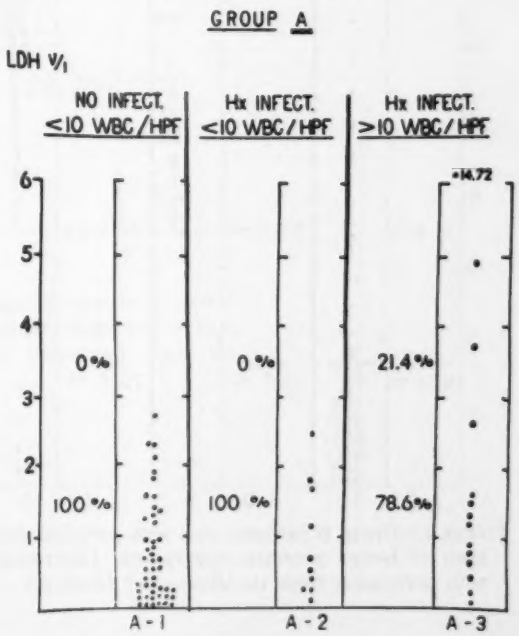

FIGURE 1.—Group A patients: men <40 years old without evidence of infection. [Reproduced with permission from the *Journal of Urology*.]

[1] Department of Urology, Northwestern University Medical School, Chicago, Ill.

[2] *Reprint requests to*: John T. Grayhack, MD, Department of Urology, Northwestern University Medical School, 303 E Chicago Ave, Chicago, Ill 60611.

TABLE 1.—Patient groups

Group	No. of patients	Age range (yrs)	Mean age (yrs)
A	72	17–39	29.4
B	70	40–79	62.3
C	15	45–87	65.9
D	129	41–82	64.4

quently present in the prostate in which malignancy has developed, and that study of the expressed prostatic fluid is a reasonable method of identifying these changes. The observations reported suggest that a study of LDH isoenzymes in prostatic fluid may be helpful in identifying patients with a high risk of prostatic malignancy.

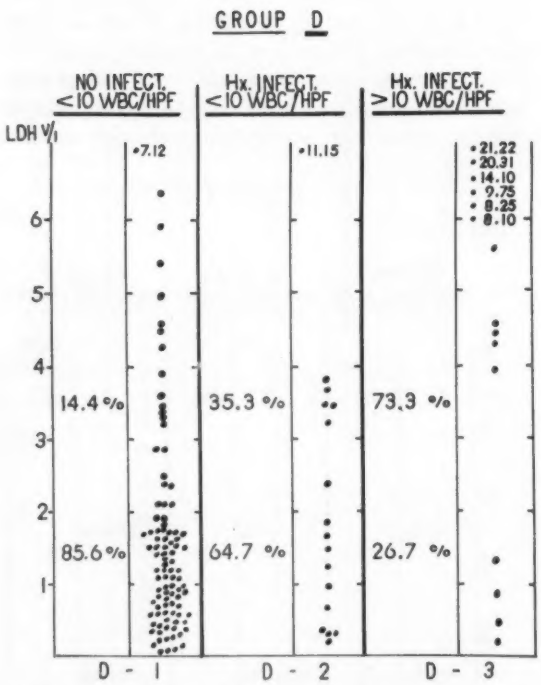

FIGURE 2.—Group D patients: men with a clinical diagnosis of benign prostatic hyperplasia. [Reproduced with permission from the *Journal of Urology*.]

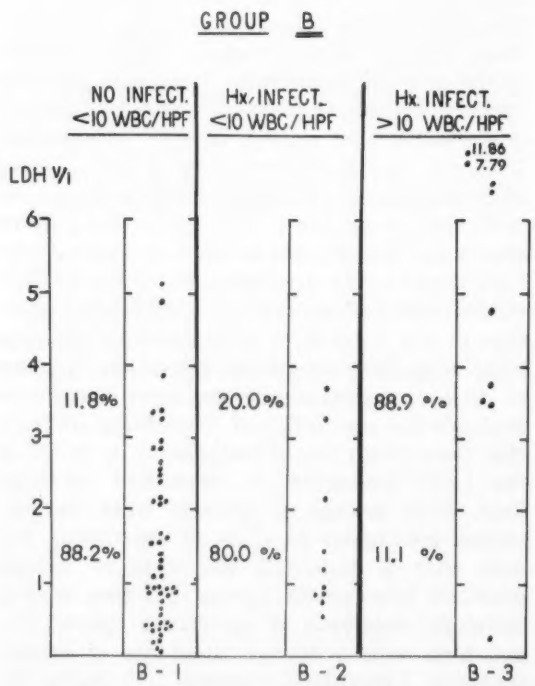

FIGURE 3.—Group B patients: men with a histologic diagnosis of benign prostatic hyperplasia. [Reproduced with permission from the *Journal of Urology*.]

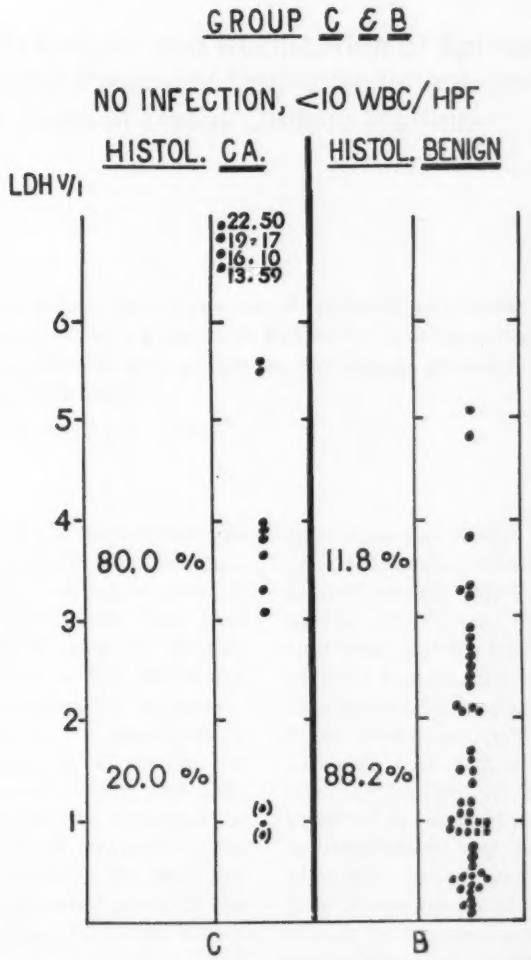

FIGURE 4.—Comparison of groups B (*see* fig 3) and C patients (men with a histologic diagnosis of carcinoma). [Reproduced with permission from the *Journal of Urology*.]

Disaggregation of Prostates and Purification of Epithelial Cells From Normal and Cancerous Prostates Using Sedimentation in an Isokinetic Density Gradient of Ficoll in Tissue Culture Medium [1]

Thomas G. Pretlow II [2,3,4]

SUMMARY

A brief summary of our work on the separation of epithelial cells from prostates and prostatic carcinomas is presented. Epithelial cells have been obtained from hamster prostates, human prostates, and human prostatic carcinomas in greater than 95% purity.

[Cancer Chemother Rep 59:143–145, 1975]

The long-term interest of our laboratory has been the development of methods for the purification of individual kinds of cells from normal and malignant human tissues. We hope that the purification of individual kinds of stromal cells from human solid tumors will be useful in the elucidation of man's response to cancer. During the past decade, there has been a marked increase in the number of studies on the interactions between cancer cells and cells which participate in the host's response to cancer. Even in the case of transplantable tumors in experimental animals, the majority of these studies have been directed towards the investigation of interactions between tumor cells and peripheral blood cells (1,2), spleen cells (3), peritoneal cells (4–8), or other readily accessible cells which are active in the immune system (9,10). In attempting to evaluate the importance of the host's lymphocytes in the case of solid tumors, we felt it would be important to evaluate the types and numbers of lymphocytes which actually arrived in the tumors. Towards this end, we recently reported (11) the purification of lymphocytes from several kinds of transplantable tumors using gradient sedimentation and adherence columns. Lymphocytes were purified more than 100-fold from suspensions of tumor cells and were obtained from a series of hepatomas, melanomas, and gliomas in a purity of 58.4%–96.8% lymphocytes.

In addition to lymphocytes, our laboratory has purified and cultured several other kinds of cells from cancers both in laboratory animals and in man (12–17). In the case of carcinoma of the prostate, purification of malignant epithelial cells from benign epithelial cells, fibroblasts, endothelial cells, inflammatory cells, and blood cells seems particularly important since the nonepithelial elements of prostatic carcinomas are frequently prominent. Similarly, if we are to compare the malignant epithelial cells with their ancestral precursors, it will be important to purify epithelial cells from the several other kinds of cells which constitute

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[2] Department of Pathology, University of Alabama, Birmingham.

[3] I would like to emphasize that much of the work reported here has been carried out by Mrs. Sherron Dow, a graduate student, and Dr. Judy Zettergren, a Research Associate, in my laboratory.

[4] *Reprint requests to*: Thomas G. Pretlow II, MD, Department of Pathology, University of Alabama, University Station, Birmingham, Ala 35294.

the majority of cells from most prostates and prostatic carcinomas.

Because of the greater expense involved in working with human tissues, our first work with the prostate was in the purification of epithelial cells from normal hamster prostates. In a series of experiments, we have obtained epithelial cells from normal hamster prostates with a mean purity of 97.2% ± 0.8%. Since we obtained funding to study human prostates and prostatic carcinomas approximately 2 months ago, we have studied the conditions which permit the purification of epithelial cells from human prostates and prostatic carcinomas. The studies which have been conducted to date suggest that a one-step purification of epithelial cells in a previously described isokinetic gradient of Ficoll (polysucrose, average molecular weight, 400,000) in tissue culture medium will result in epithelial cells having a purity approximately equivalent to the purity which was obtained in work with the hamster prostate. This work is still rather embryonic, and we shall need to study many more human prostates with and without cancer before we can be certain that the conditions which we have developed are broadly applicable to prostates with and without carcinoma from a large number of patients.

At the present time, it appears that the actual purification of the epithelial cells is relatively simple after cells are obtained in suspension from the human material. The most unsatisfactory part of our procedure is the actual process of obtaining the cells in suspension. In 1970, Franks et al (18) reported that prostatic epithelial cells which were separated from prostatic stromal elements by mechanical means showed a "failure to survive and proliferate" in culture. Using several mechanical methods for obtaining prostate cells in suspension, we have found that a large proportion of mechanically suspended cells do not exclude trypan blue. We have obtained a high proportion of cells which exclude trypan blue following the dissociation of human prostates with 0.1% pronase (EM Laboratories, Elmsford, NY). Similarly, high proportions of viable cells have been obtained from prostatic carcinomas using 0.25% trypsin (Grand Island Biological Co., Grand Island, NY) and 0.1% pronase. In the case of the hamster prostate, we obtained 25.7 ± 8.7 × 10^6 cells/g of tissue. From human prostates, we have obtained less than one tenth as many cells per gram. More efficient methods for obtaining cell suspensions from human prostates and prostatic carcinomas are needed, and we are currently evaluating several other enzymes and chelating agents in our attempt to obtain large numbers of suspended, viable cells from human prostates and prostatic carcinomas.

Because pure fractions of malignant cells from human prostatic carcinomas have not been available previously, investigations of the biochemistry, immunochemistry, and virology of carcinoma of the prostate have been dependent on tumors in which the malignant cells were diluted with more than an equal number of stromal fibroblasts, inflammatory cells, endothelial cells, and blood cells. We hope that the development of methods for purifying epithelial cells from prostatic carcinomas and from prostates will facilitate the more specific characterization of the malignant prostatic cells and their normal ancestral precursors.

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Cultivation of Epithelial Cells From the Prostate [1]

N. R. Rose, B.-K. Choe, and J. E. Pontes [2,3]

SUMMARY

We have (*a*) examined culture conditions for prostatic cells, (*b*) devised a procedure for the selective enrichment of epithelial cells, and (*c*) developed a way to identify prostatic epithelial cells by immunologic methods.

[Cancer Chemother Rep 59:147–149, 1975]

We are trying to develop an immune cytotoxicity assay for prostatic cancer. As a target cell for such an assay we have been culturing prostatic biopsy or surgical specimens from patients with benign hypertrophy and cancer. In culturing prostatic cells, we have the following goals in mind: (*a*) to define the optimal growth conditions for primary culture of prostatic cells; (*b*) to devise a method of selective enrichment of epithelial cells; and (*c*) to identify these epithelial cell populations as the proper target cells on the basis of enzymologic and immunologic criteria.

We have been able to initiate prostatic primary cultures from eight cases of benign hypertrophy and two cases of cancer without a single failure. Two methods were tried: (*a*) dissociation of minced tissue in trypsin-pronase buffer solution and plating of dispersed single cells; or (*b*) explant culture of finely sliced (approximately 1–2 mm^3) tissue. We have adopted the latter method as the standard. We tried several combinations of commercially available tissue culture media: (*a*) Eagle's minimal essential medium with nonessential amino acids and sodium pyruvate; (*b*) RPMI 1640; and (*c*) Ham's F12. All of these media were supplemented with 25% fetal calf serum and supported good growth of primary cultures. From the outgrowth of explants we could harvest approximately 3×10^5 cells within 3 weeks. Figure 1 shows outgrowth from prostate explants. Both fibroblasts and epithelial cells can be seen.

To obtain epithelial cells from these mixed populations we have examined various conditions of trypsinization. As shown in table 1, simple trypsinization caused detachment of prostatic fibroblasts from the glass wall of the culture flask within 20–30 minutes, whereas epithelial cells detached very slowly. There was pronounced clumping of the epithelial cells. Preincubation of monolayers in EGTA [4] buffer (Ca^{++}, Mg^{++} free) and subsequent incubation of cells in trypsin-pronase mixtures or trypsin-pronase-collagenase mixtures gave complete detachment and excellent dispersion of epithelial cells. This selective trypsinization method enabled us to enrich prostatic epithelial cells from explant outgrowths. For the recovery of viable cells with high plating efficiency, it is important to wash the dispersed cells with growth medium before plating. Adequate dissociation of epithelial cells was found to be necessary for the storage of these cells under liquid nitrogen.

Differentiation of fibroblasts from epithelial cells was done on a morphologic basis. Also, as

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[2] Departments of Immunology and Microbiology, and of Urology, Wayne State University School of Medicine, Detroit, Mich.

[3] *Reprint requests to:* Dr. N. R. Rose, Wayne State University School of Medicine, Detroit, Mich 48201.

[4] EGTA = ethyleneglycol-bis-(β-amino ethyl ether)-*N*, *N'*-tetraacetic acid.

TABLE 1.—Dissociation of epithelial cells from mixed prostatic cell cultures

Dispersing method*	Efficiency of detachment (%)	Viability by dye exclusion (%)	Plating efficiency (%)	Comment
A	1–2	50–60	<30	Poor detachment and clumping of epithelial cells; complete detachment of fibroblasts
B	100	95	86	Complete detachment without clumping
C	100	98	93	Complete detachment without clumping

*A = trypsin: monolayers are washed with saline and trypsinized with 0.25% trypsin for 10–30 mins at 37°C.

B = trypsin + pronase: monolayers are incubated in 100 μmols of EGTA–Hank's balanced salt solution for 15 mins at 37°C and subsequently dissociated for 5 mins in 0.125% trypsin-0.05% pronase-EGTA-Hank's balanced salt solution supplemented with 2.5% serum at 37°C. Before plating, detached cells are washed with 10 volumes of growth medium.

C = trypsin + pronase + collagenase: the same as B except that 0.05% collagenase is included.

can be seen from table 2, histochemical staining is valuable for the identification of cell types. Epithelial cells stain strongly for acid phosphatase, while fibroblasts are initially negative. However, prolonged incubation gives positive staining for the fibroblasts also. We looked into the possibility of exploiting specific antigens expressed only in the prostatic epithelial cells for differentiation of prostate from other cells. One of the first candidates to be examined is prostate-specific acid phosphatase. For that reason we have purified acid phosphatase from several tissues, including prostatic fibroblasts, serum, liver, prostate, and semen. The protocol used is shown in figure 2. The molecular weights of prostatic acid phosphatase isoenzymes were determined and immunologic specificity of acid phosphatases from several tissues was examined. Antiprostatic acid phosphatase monkey serum was then purified by $(NH_4)_2SO_4$ precipitation followed by DEAE-cellulose column chromatography. As can be seen from figure 3, the IgG fraction of absorbed antiserum eluted from an acid phosphatase immunoabsorbent column specifically recognized prostatic acid phosphatase. Such an IgG fraction was coupled to fluorescene isothiocyanate (FITC). Slide cultures of cells of prostatic or nonprostatic origin were stained with antiprostatic acid phosphatase IgG-FITC conjugate. Under these conditions, only prostatic epithelial cells from patients with benign hypertrophy or cancer showed positive immunofluorescence (table 2).

TABLE 2.—Examination of cell cultures for acid phosphatases

Cells	Acid phosphatase demonstrated by: Histochemical staining*	IF†
Lung fibroblasts (WI-38)	+	−
Prostatic fibroblasts	+	−
Prostatic epithelial cells		
Benign hypertrophy	++	+
Cancer	++	+

*Naphthol AS-MX phosphoric acid (10 mg) and garnet GBC (10 mg) in 0.2 M acetate buffer (pH 5.5, 20 ml) for 30 mins at 37°C.

†IF = immunofluorescence technique. Specific antibody fraction was prepared from anti-prostatic acid phosphatase serum by $(NH_4)_2SO_4$ precipitation, DEAE-cellulose column chromatography, and immunoabsorbent column chromatography. The IgG fraction was conjugated to FITC and the specificity of resulting antiserum-FITC conjugate was verified by Ouchterlony double diffusion.

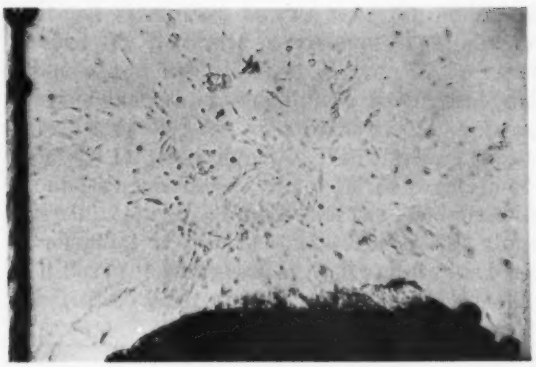

FIGURE 1.—Primary culture of human prostatic cancer cells grown in Eagle's minimal essential medium supplemented with nonessential amino acids, sodium pyruvate, and 25% fetal calf serum. Cells are mostly epithelial, and some have vacuoles and lipid inclusions (unstained, standard optics, × 100).

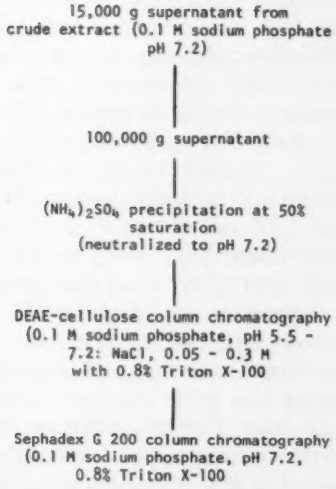
15,000 g supernatant from crude extract (0.1 M sodium phosphate pH 7.2)
100,000 g supernatant
$(NH_4)_2SO_4$ precipitation at 50% saturation (neutralized to pH 7.2)
DEAE-cellulose column chromatography (0.1 M sodium phosphate, pH 5.5 - 7.2: NaCl, 0.05 - 0.3 M with 0.8% Triton X-100
Sephadex G 200 column chromatography (0.1 M sodium phosphate, pH 7.2, 0.8% Triton X-100

FIGURE 2.—Preparation of acid phosphatases from various tissues (protein, enzyme activity, and immunologic specificities are examined at each step).

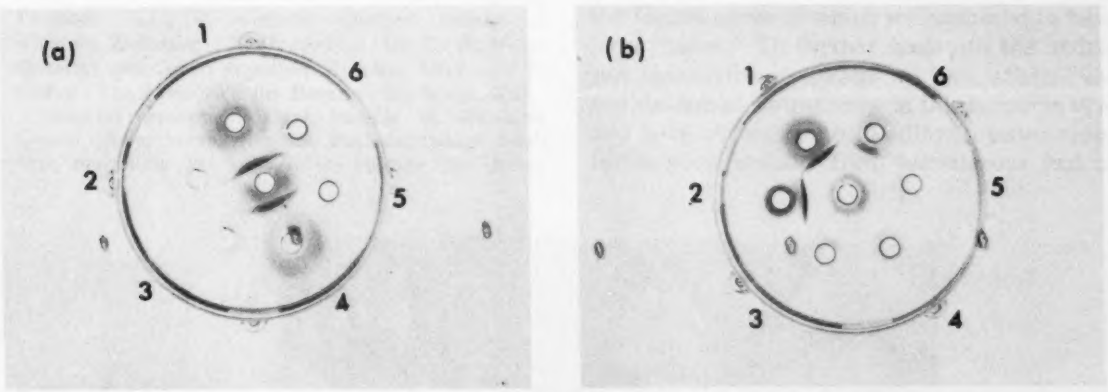

FIGURE 3.—Immunologic specificity of monkey antiserum to acid phosphatase (AcP). (*a*) Center well, monkey antiserum to human prostate; wells 1 and 4, AcP from human prostate; wells 2 and 5, AcP from WI-38; wells 3 and 6, AcP from HeLa cells. (*b*) Center well, human semen AcP; well 1, unabsorbed monkey antiserum; well 2, antiserum absorbed with normal human serum; well 3, antiserum absorbed with crude AcP fraction from WI-38; well 4, antiserum absorbed with crude AcP fraction from HeLa cells; well 5, antiserum absorbed with human prostate extract; well 6, antibodies eluted from immunoabsorbent column made of prostatic AcP.

General Discussion: Detection and Diagnosis of Prostatic Cancer [1,2]

W. Voigt: In this session we have heard several references to the need for an animal model for prostatic carcinoma, especially one that would exhibit some degree of hormonal responsiveness. I would like to present data pertaining to such an animal model. Approximately 11 years ago Dr. Wilhelmina Dunning found a spontaneous tumor in the prostate of a Copenhagen rat and she had the good vision to transplant it to other rats. Today, the tumor (R-3327) is still growing well and we have now characterized it for its responsiveness to hormones and endocrinologic manipulation. Figure 1 shows that histologically the tumor still remains an adenocarcinoma; well-differentiated glands with hyperplastic epithelium can be seen. In the center of the acini you can see an abundant secretion. Figure 2 shows the growth of R-3327 in males, females, and castrated males. The tumor diameter was plotted against the days after implantation. The figure shows that the tumor grew much better in males than in females but grew very little in castrated males. These data are somewhat deceptive because the curve in the males should really be much steeper. The problem is that we inoculate two tumors on both sides and sometimes one of these explants doesn't take at all while the contralateral tumor grows extremely well. While this is obviously not a hormonally related problem, for statistical accuracy we have averaged all sites of implantation in our points and this makes this curve somewhat closer to the female curve in which we happened to have more "takes." To further ascertain the androgen sensitivity of R-3327 we have studied the metabolism of testosterone in this tumor in vivo and have observed that 5α-dihydrotestosterone forms preferentially from testosterone just as

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[1] The following investigators participated in this discussion: *W. Voigt* (University of Miami School of Medicine, Fla), *P. Guinan* (Cook County Hospital, Chicago, Ill), *T. Chu* (Roswell Park Memorial Institute, Buffalo, NY), *R. Van Etten* (Purdue University, Lafayette, Ind), *A. Seligman* (Sinai Hospital of Baltimore, Inc., Md), *B. Mobbs* (University of Toronto, Canada), *M. Tannenbaum* (Columbia University, New York, NY), *D. Byar* (National Cancer Institute, Bethesda, Md), *C. Moncure* (Medical College of Virginia, Richmond), *M. Maruyama*, (M. D. Anderson Hospital and Tumor Institute, Houston, Tex), and *D. Coffey* (The Johns Hopkins Hospital, Baltimore, Md).

[2] *Reprint requests to:* Mrs. Pamela W. Hutchins, *Cancer Chemotherapy Reports*, National Cancer Institute, Blair Bldg, Rm 3A05, Silver Spring, Md 20910.

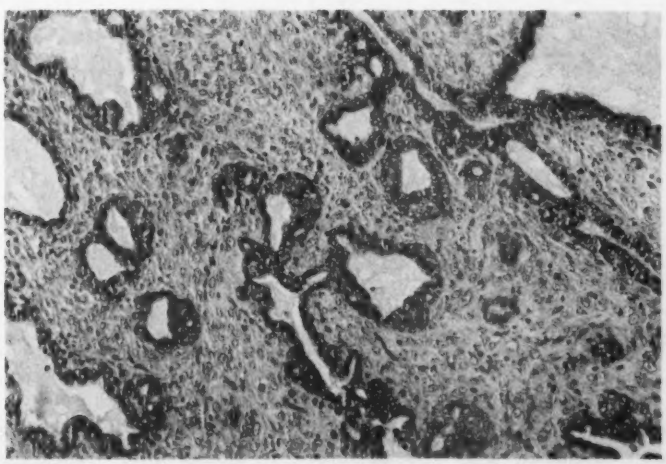

FIGURE 1.—Histology of prostatic adenocarcinoma (R-3327) of a Copenhagen rat.

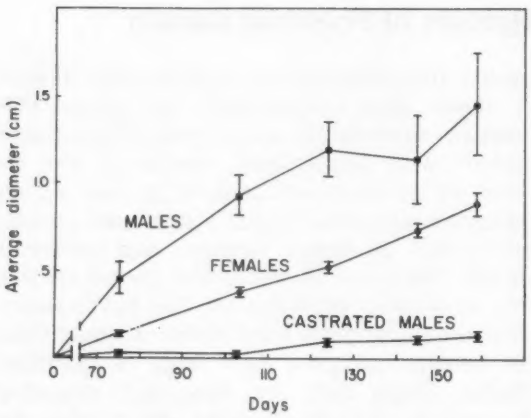

FIGURE 2.—Growth of R-3327 in male, female, and castrated male Copenhagen rats.

in the normal rat prostate. This is in contrast to what happens in non-androgen-responsive tissue such as muscle or liver, in which the main metabolites are oxidized products such as 4-androstenedione and some other more polar metabolites. The presence of androgen receptors in R-3327 has been detected by binding studies with 5α-dihydrotestosterone. Figure 3 shows a sucrose gradient centrifugation of the tumor cytosol after incubation with ^{3}H-5α-dihydrotestosterone. A separate line has been derived from R-3327 which has lost its androgen dependency. This line, called the A-series, grows equally well in females, castrates, or males. Figure 4 shows that in contrast to R-3327, the A-series tumors have lost the receptors for androgens, which explains their androgen insensitivity. By this technique we would hope that by taking specimens at the time of surgery or transurethral resection, we would be able to predict which prostatic carcinomas would be

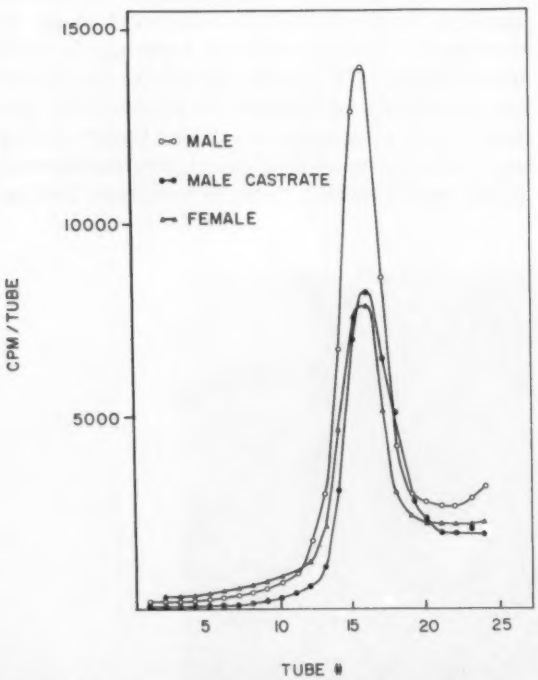

FIGURE 3.—Sucrose gradient centrifugation of R-3327 tumor cytosol after incubation with ^{3}H-5α-dihydrotestosterone (^{3}H-DHT).

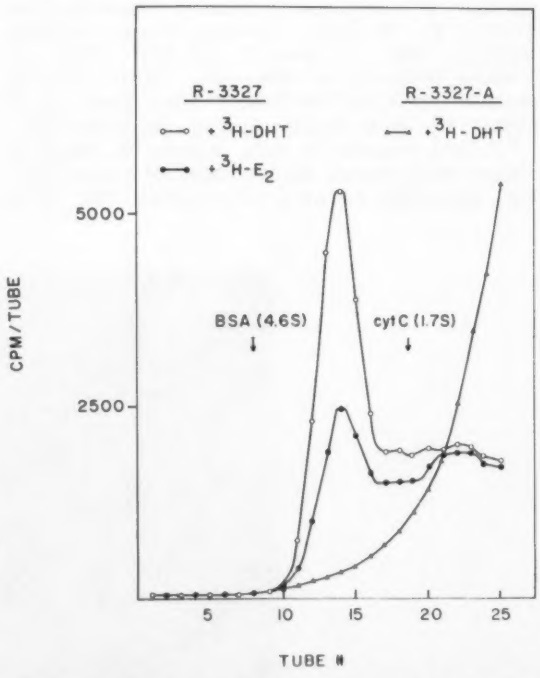

FIGURE 4.—Sucrose gradient centrifugation of A-series tumors after incubation with ^{3}H-5α-dihydrotestosterone (^{3}H-DHT).

amenable to endocrine manipulation, such as castration or hormonal therapy, thereby avoiding some unnecessary surgery in those patients whose tumors are not androgen dependent.

P. Guinan: I would like to comment briefly on the paper by Chu et al (1), but only to add a note of caution in the use of the carcinoembryonic antigen (CEA) test in evaluating and following patients with carcinoma of the prostate. Table 1 is a summary of the literature on the CEA accuracy in carcinoma of the prostate. As you can see, 157 patients were evaluated in these four papers; 78 were positive for an overall accuracy of 49%. Again, these figures were not broken down as to stage or therapy but they do indicate that less than 50% of the patients were found to be CEA positive and, as Dr. Chu mentioned, the upper limit of normal was 2.5 ng. We've continued this study at Cook County Hospital, Chicago, Illinois, and have now evaluated 954 determinations from 187 patients. Only 680 of these have been blood determinations, however, and 266 (39%) were positive (table 2). Interestingly enough, 14% of the urine tests were positive but many of those were infected, so that further evaluation is required. In breaking down the blood CEAs into active and inactive diseases there are about an equal number of determinations. More positives were found in patients with active disease (49%) than in patients considered to have inactive disease (30%). This 49% corresponds to the cumulative percent positive in the literature. In addition to this we followed some patients with serial CEA, which is felt to be prognostic in colon carcinoma as evaluated by Holyoke (2). We evaluated 76 patients with prostatic carcinoma (table 3). Seventeen had normal CEAs, 21 had a rise in serial CEAs, and 28 had decreasing serial CEAs. Disease status is shown for these patients. In summation, the misleading serial CEAs (that is, rising CEAs with inactive disease [61%] or falling CEAs with active disease [59%]) give an error of 60%. I think further evaluation of CEA in prostatic cancer is indicated.

TABLE 1.—CEA accuracy in carcinoma of the prostate

Author and year	Reference No.	No. of patients	No. of positive evaluations	Percent
LoGerfo (1971)	3	52	27	48
Reynoso (1972)	4	28	7	25
Laurence (1972)	5	9	4	44
Guinan (1973)	6	68	40	59
Totals		157	78	49

TABLE 2.—CEA accuracy in carcinoma of the prostate (Cook County Hospital, 187 patients)

Test performed on—	No. of determinations	No. of positive evaluations	Percent
Blood	680	266	39
Urine	274	38	14
Totals	954	304	

TABLE 3.—Serial CEA and status of disease

Serial CEA	No. of patients	Status of disease	No. of patients
Normal	17	Active	3
		Inactive	14
Rising	31	Active	12
		Inactive	19
Falling	28	Active	16
		Inactive	12
Totals	76		76

T. Chu: I would just like to clarify one point. The data I showed on the CEA in my presentation were primarily from prostatic fluid and I did not discuss the CEA in plasma which has been published elsewhere. I hope I did not confuse the issue.

R. Van Etten: Dr. Kaighn, Dr. Rose, and others have been discussing prostatic acid phosphatase and questions have been raised about the significance of the serum acid phosphatase level as a unique diagnostic indicator. Work that we are doing in this area might be of interest. We have been comparing human liver acid phosphatase with human prostatic acid phosphatase. We have achieved an 80-fold purification of an isoenzyme from human liver and find that it is remarkably similar to human prostatic acid phosphatase. It has a molecular weight somewhat less than 100,000, it is tartrate inhibitable, it has a similar pH dependence, it is strongly inhibited by molybdate ion and certain other related ions, and, to the extent that we have examined, its substrate specificity is also similar. I think that there should be considerable caution in equating serum acid phosphatase levels with prostatic acid phosphatase levels. This also, of course, opens many new areas of investigation. We have talked about some very interesting possible experiments with Dr. Moncure and I'm sure that many can think of other related things to do.

A. Seligman: I would like to make a statement about the repeated references to acid phosphatase I've heard mentioned today, many times without indicating what substrate was used. I think the understanding seems to be prevalent that all acid phosphatases are alike. They are not alike and there are differences in substrate specificity which are presented in my talk (7).

B. Mobbs: For about a year, we have been attempting to predict endocrine responsiveness in patients with prostatic carcinoma using dihydrotestosterone binding as a predicting mechanism. We have found that there is a large variation in dihydrotestosterone binding by the carcinoma specimens, and we are trying to relate this to response in the patients receiving hormonal treatment. We are concerned that some of these specimens may be contaminated with testosterone-binding globulin which is high in patients who are receiving estrogens: this globulin has, of course, a very high affinity for dihydrotestosterone and could confuse the assay. This problem doesn't arise in the rat because the rat has no testosterone-binding globulin.

M. Tannenbaum: I would like to ask Dr. Mostofi or Dr. Byar a question that relates to Dr. Gleason's work (8) which was mentioned. In the material you have histologically examined is there any statistical correlation between the major and minor patterns (described by Dr. Gleason) found in your needle biopsies or transurethral resection material with that taken out later during the radical prostatectomy or autopsy? If there is a statistical correlation, then we should be able to predict in advance what might be found later in surgical or autopsy specimens. The surgical pathologist is faced with this perplexing problem every day.

D. Byar: Dr. Gleason's primary and secondary patterns refer only to the amount of each pattern present in the biopsy. You can never find the same thing in later biopsies that you found the first time because you took it out. This is a fundamental problem of looking at serial biopsies and expecting to see progression of tumor dedifferentiation. With respect to autopsies, I don't know if you were referring to the grade of the tumor at autopsy or to the distribution of metastases at autopsy. In the latter case, we have reviewed autopsy protocols from some 300 patients, all of whose tumors were graded at the time of diagnosis. In every major site where cancer is found at autopsy the probability that that site will contain metastasis depends strongly on the initial grade of the tumor. The relationship is remarkably simple and clear.

C. Moncure: I would like to reiterate that there is certainly more than one acid phosphatase, and I think maybe we should be investigating phosphatases other than prostatic in other disorders. The last slide I showed in my presentation (9) showed negative bone marrow studies from a patient with Gaucher's disease. In a student project we had the opportunity to immunize two rabbits with a spleen from a patient with Gaucher's disease (fig 5). That spleen is the well on the left in the figure. The two antisera are the wells on the top and the bottom, and the prostate is on the right. Both of these rabbits developed two anti-acid phosphatases against Gaucher's cell, one of which in the bottom well shows a slight curving, possibly indicating a partial identity with the prostate. I'd like to re-emphasize that we do need to look into other lysosomal acid phosphatases, and I don't believe it's all just a matter of the carbohydrate side groups. I think we're probably dealing with genuine differences in the polypeptide chains.

K. Maruyama: In connection with Dr. Theodore Malinin's presentation, I would like to make a comment. As he pointed out, we have received some of the tissues from his tissue bank. One of them was received completely thawed. We thought it might not be of any use

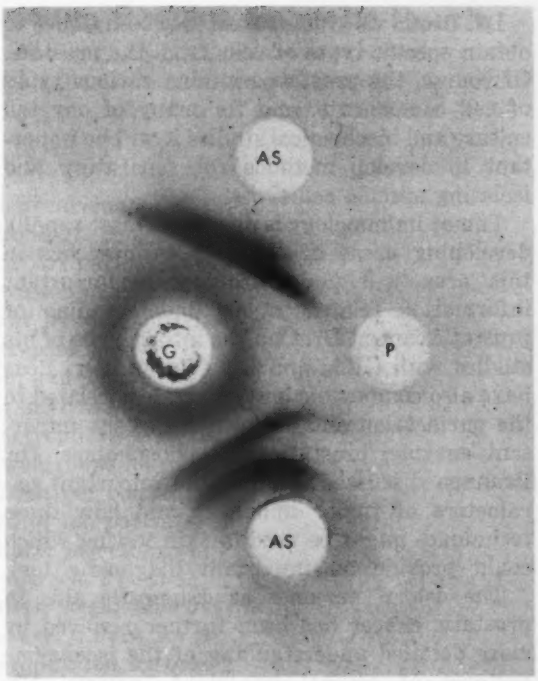

FIGURE 5.—Anti-acid phosphatases developed in rabbits against Gaucher's cell. AS = antisera; G = spleen from patient with Gaucher's disease; P = prostate.

for tissue culture but felt we might as well process it. Indeed, we were proved wrong and Dr. Malinin was right. Both epithelial and fibroblastic cell cultures were obtained from a human fetal prostate specimen.

D. Coffey: I believe we should attempt to place this session in focus by a quick review of what has been accomplished. The working cadre of the National Prostatic Cancer Project recognizes the importance of having a large supply of prostatic tissue available for study. It is difficult for many investigators to obtain these samples and Dr. Malinin has extensive experience in cryobiology related to the storing and maintenance of viable tissue banks. He has summarized the facilities and techniques which are available and has already initiated the collection of human prostatic cancer tissue from several centers. This should provide a valuable resource to investigators interested in the study of this disease.

Dr. Pretlow's studies described techniques to obtain specific types of cells from the prostate. Of course, the prostate contains various types of cell components, and for many of our cell culture and biochemical studies it will be important to develop methods for separating and isolating specific cell types.

Tumor immunology is one of the most rapidly developing areas of oncology. Approaches in this area will undoubtedly yield important information related to our understanding of prostatic cancer. Dr. Chu has informed us of his studies with CEA, and Dr. Chu and Dr. Rose have also commented on their studies related to the purification and antigenicity of the important enzyme, prostatic acid phosphatase. Dr. Brannen discussed some of the important parameters of tumor immunity and how these techniques might be used in skin testing which could provide an important diagnostic tool.

The use of enzymes as diagnostic aids in prostatic cancer has been further resolved by more detailed understanding of the isoenzyme patterns of several important systems. Dr. Moncure has reviewed several of these systems and Dr. Grayhack has discussed how the isoenzyme ratios of lactic acid dehydrogenases can be related to the status of prostatic cancer.

Dr. Hadd has informed us of his studies and plans to use radiolabeled androgens as a means of developing radioscanning techniques to delineate prostatic tissue.

Dr. Mostofi reviewed the important histologic parameters of prostatic cancer and stressed the many varied types of prostatic adenocarcinoma.

In summary, we have reviewed some of the new findings and approaches that might yield new methods of detecting and diagnosing early prostatic cancer. These findings will undoubtedly contribute to a better understanding of this disease process and how it might be controlled. It is apparent that any one of these techniques might not be sufficient, but the combination of immunologic studies, isoenzyme patterns, and new and improved scanning techniques could possibly yield more precise diagnosis and better insight into the prognosis and therapy of this disease. We are at the initial stages of many of these studies and I am sure that each of us looks forward to monitoring these developments in hopes that they will provide the much needed clinical tools to apply to this devastating disease of prostatic cancer.

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Immunotherapy of Prostatic Carcinoma With Bacillus Calmette-Guerin

C. Merrin, T. Han, E. Klein, Z. Wajsman, and G. P. Murphy [1,2]

SUMMARY

Seventeen patients with histologically proven adenocarcinoma of the prostate were selected for evaluation of their immunologic competence and therapy with bacillus Calmette-Guérin (BCG). All patients were in stage D. The immune response was explored in two main aspects: cell-mediated and humoral immunity. Delayed skin hypersensitivity reaction with purified protein derivative (PPD), streptokinase-streptodornase (varidase), Candida, mumps antigen, and Trichophyton were tested. Lymphocyte reactivity was measured by in vitro blastogenesis. Serum immunoglobulin levels and serum protein electrophoresis were also measured. The patients were then divided in two groups according to the skin test response to PPD. Group 1 (PPD-positive) consisted of seven patients. Group 2 (PPD-negative) consisted of ten patients. In group 1, two patients were treated with intraprostatic injection of BCG every week in the following doses: 1 cc the first week, 2 cc the second week, 4 cc the third week, and 6 cc the fourth week. The five remaining patients in this group received only 1 cc every week for 4 weeks. The group 2 patients (PPD-negative) were stimulated by oral intake of BCG in an attempt to convert their skin tests to positive. All patients revealed varying degrees of immunodepression. None of the patients in group 2 (stimulated by oral intake of BCG) converted to positive skin tests. Three patients in group 1 treated with BCG showed necrosis of the tumor. The different aspects of immunodepression in this disease are analyzed and correlated to the clinical staging, histologic grading, and response to therapy. The mechanism of BCG action in advanced prostatic carcinoma is discussed.

[Cancer Chemother Rep 59:157–163, 1975]

In the past 200 years physicians have observed a curious phenomenon: the regression of some malignant tumors when associated with severe bacterial infections. The first physician to translate this observation to medical investigation was W. B. Coley, who from 1891 to 1936 produced more than 100 papers on the treatment of malignant neoplasm by intratumor injection of bacteria.

Since then, numerous investigations in animals and man have demonstrated that when in contact with tumors bacterial elements may produce a nonspecific stimulation of the immune system with eventual destruction of these tumors. This has been clinically illustrated by Mathé et al (1–5) in France.

At Roswell Park Memorial Institute, Sokal et al (6–9) have treated leukemias and lymphomas with good results, and Klein et al (10–12) have had spectacular remissions in cancer of the skin and melanomas. Their efforts and results have encouraged us to investigate this approach in the treatment of carcinoma of the prostate. Our results are presented herein.

[1] Roswell Park Memorial Institute, New York State Department of Health, and the State University of New York, Buffalo.

[2] *Reprint requests to*: Claude Merrin, MD, Roswell Park Memorial Institute, 666 Elm St, Buffalo, NY 14203.

SELECTION OF PATIENTS

Seventeen patients were entered in the study at Roswell Park Memorial Institute from September 1972 to May 1974. All patients were referred for treatment by other urologists, had failed hormonal therapy, had histologically proven adenocarcinoma of the prostate, and were in stage D. Patients' ages ranged from 51 to 84 years with an average of 67 years. All those studied had measurable radiologic lesions of malignant disease. All patients were followed until their death. At present, the average followup is 8 months.

EVALUATION

The patients were evaluated by physical examination, intravenous pyelogram, chest roentgenogram, bone survey, liver and bone scans, cystoscopy, prostate biopsy, and routine biochemical and hematologic studies.

Both cell-mediated and humoral immunologic responses were examined in these patients. Cell-mediated immunity was tested by skin reactions and in vitro lymphocyte blastogenesis. Delayed skin hypersensitivity reactions with purified protein derivative (PPD), streptokinase-streptodornase (varidase), Candida, mumps antigen, and Trichophyton were observed at 24 and 48 hours. A reaction was considered positive when the size of the erythema and induration was 1 cm or more in diameter. Any smaller reaction was considered negative. Lymphocyte reactivity was evaluated by in vitro blastogenic response to phytohemagglutinin concanavalin A and pokeweed mitogen using the ^{3}H-thymidine-incorporation method. Lymphocyte response was expressed as a blastogenic index which is a ratio of the counts per minute measured in stimulated cultures and the counts per minute in unstimulated cultures. Humoral immunity was tested by serum protein electrophoresis and serum immunoglobulin level. The patients were divided into two groups. Group 1 had positive PPD skin tests and group 2 had negative PPD skin tests. Seven patients were in group 1 and ten patients were in group 2. Patients in group 2 received 50 cc (50 mg) of bacillus Calmette-Guérin (BCG) orally every week for 2 weeks to convert their negative PPD skin tests to positive. Skin tests were repeated every week for 3 weeks.

In group 1, two patients were injected in the prostate with BCG: 1 cc the first week, 2 cc the second week, 4 cc the third week, and 6 cc the fourth week. The other patients in the same group received 1 cc/week of BCG in the prostate for 4 weeks. (There was 1 mg of dry weight of BCG per cubic centimeter.)

Perineal biopsies were taken at 2, 4, 6, and 8 weeks. Complete blood cell counts and chemistry tests were done every week. Immunoglobulin levels (IgG, IgA, and IgM) were determined every 2 weeks. Bone surveys were done at 4 and 8 weeks.

A system was constructed to define the different degrees of response. Objective response was defined as a 50% decrease of a measurable lesion. Stabilization was defined as no change in the size of lesions or the symptoms. Subjective responses were also defined as a decrease of pain and a general sensation of well-being.

RESULTS

In group 1 (table 1) four patients had a normal lymphocyte activity as expressed by the blastogenic index. Three patients had decreased blastogenic indexes. The seven patients in this group showed hyporeactive skin tests. Several abnormalities in the serum protein electrophoresis were detected. Two patients had an increase in α_1-albumin, three patients had an increase in β-globulins, one patient had an increase in α_2-albumin, and one patient had an increase in γ-globulins. Four patients had normal serum protein electrophoresis. Two patients had an increase in IgG immunoglobulins, one patient had an increase in IgM immunoglobulins, and one patient had a decrease in IgG immunoglobulins. The other three patients had normal immunoglobulin levels. Lymphocyte counts were normal in five patients, elevated in one patient, and decreased in one patient.

In group 2 (PPD-negative) (table 2) two patients had a normal lymphocyte blastogenic index and eight patients had a decrease in lymphocyte blastogenic activity. Skin tests were negative in six patients and hyporeactive in four patients. Serum protein electrophoresis

TABLE 1.—Immune response before treatment with BCG: group 1 (PPD-positive)

Patient No.	Blastogenic index	Skin tests*	Protein electrophoresis	Immunoglobulin	Lymphocyte count
1	Decrease	Hyporeactive (1/5)	Increase in α_1-albumin and β-globulin	Increase in IgG	Normal
2	Decrease	Hyporeactive (3/5)	Increase in α_1-albumin, β-globulin, and α_2-albumin	Increase in IgM	Normal
3	Normal	Hyporeactive (1/5)	Normal	Normal	Normal
4	Normal	Hyporeactive (1/5)	Increase in β- and γ-globulins	Increase in IgG	Increase
5	Normal	Hyporeactive (1/5)	Normal	Normal	Normal
6	Normal	Hyporeactive (1/5)	Normal	Normal	Normal
7	Decrease	Hyporeactive (1/5)	Normal	Decrease in IgG	Decrease

*Nos. in parentheses = No. of positive skin tests over No. of tests performed.

was normal in six patients and showed some abnormalities in four patients. Of these, two patients had an increase in β-globulin levels, two patients had an increase in α_2-albumin, and one patient had an increase in γ-globulins. Immunoglobulins were normal in seven patients, two patients had an increase in IgG immunoglobulins, and one patient had an increase in IgM immunoglobulins. Lymphocyte counts were normal in eight patients and decreased in two patients.

None of the patients in group 2 (PPD-negative) who received BCG orally had PPD skin tests converted to positive.

The patients in group 1 (table 3) who were treated with intraprostatic injections of BCG showed the following: five patients had objective responses; five patients had a decrease in the size and induration of the prostate; and one patient had tumor necrosis in the prostate and, as a consequence, a rectoprostatic fistula developed. No changes were observed in measurable metastases with the exception of one patient who had a soft tissue metastasis injected with BCG. This metastasis showed tumor necrosis. The general condition became worse in four patients. It remained stable in two patients and improved in one patient. The immune response after therapy was analyzed (table 4). This response remained unchanged in two patients, improved in three patients, and decreased in two patients. Two patients had normalization of their serum protein electrophoresis. The same two patients had normalization of serum immunoglobulin levels and two patients showed an increase in IgG. The skin tests remained unchanged in six patients and became negative in one. Two patients who previously had a normal blastogenic index showed some decrease in lymphocyte activity. Lymphocyte counts which were elevated in one patient became normal.

Pathology Studies

Analysis of the biopsies taken after BCG injections revealed necrosis of the tumor in three patients and no changes in four patients. There was no evidence of lymphocytic infiltration or granuloma formation at the site of the injection (figs 1–3).

Side Effects

The first two injections were well tolerated but the third and fourth injections produced fever in all patients. These symptoms and signs responded well to treatment with aspirin and hydrazide of isonicotinic acid (table 5). Two patients had an anaphylactic-like reaction to the fourth injection. They responded well to treatment with benadryl and high doses of steroids.

DISCUSSION

All patients in this study had varying degrees of immunodepression as expressed by hyporeactive skin tests and a decrease in lymphocyte

TABLE 2.—Immune response before treatment with BCG: group 2 (PPD-negative)

Patient No.	Blastogenic index	Skin tests	Protein electrophoresis	Immunoglobulin	Lymphocyte count
1	Normal	Negative	Normal	Normal	Normal
2	Decrease	Negative	Increase in α_2-albumin and β-globulin	Increase in IgG	Normal
3	Decrease	Negative	Normal	Normal	Decrease
4	Normal	Hyporeactive	Increase in β-globulin	Normal	Normal
5	Decrease	Negative	Increase in α_2-albumin	Normal	Normal
6	Decrease	Negative	Normal	Normal	Normal
7	Decrease	Hyporeactive	Increase in γ-globulin	Increase in IgG	Normal
8	Decrease	Negative	Normal	Normal	Normal
9	Decrease	Hyporeactive	Normal	Increase in IgM	Decrease
10	Decrease	Hyporeactive (2/5)*	Normal	Normal	Normal

*No. of positive skin tests over No. of tests performed.

TABLE 3.—Response to therapy with intraprostatic injection of BCG in carcinoma of the prostate

Patient No.	Prostate		Metastases	General condition	Immune response
	Size	Induration			
1	Decrease	Decrease	No change	Worse	Improved
2	Decrease followed by increase	Decrease	No change	Worse	Decreased
3	Decrease	Decrease	No change	Worse	No change
4	Decrease	Decrease	No change	Improved	Improved
5	No change	No change	No change	Stable	No change
6	Decrease	Decrease	Decrease followed by no change	Worse	Improved
7	No change	No change	No change	Stable	Decreased

TABLE 4.—Immune response after treatment with BCG: group 1

Patient No.	Blastogenic index	Skin tests*	Protein electrophoresis	Immunoglobulin	Lymphocyte count
1	Decrease	Hyporeactive (1/5)	Normal	Normal	Normal
2	Decrease	Hyporeactive (1/5)	Normal	Normal	Normal
3	Decrease	Negative	Increase in α_2-albumin	Increase in IgG	Normal
4	Decrease	Hyporeactive (1/5)	Increase in γ- and β-globulins	Increase in IgG, IgA, and IgM	Normal
5	Normal	Hyporeactive (1/5)	Normal	Normal	Normal
6	Normal	Hyporeactive (1/5)	Normal	Increase in IgG	Normal
7	Decrease	Hyporeactive (1/5)	Normal	Normal	Decrease

*Nos. in parentheses = No. of positive skin tests over No. of tests performed.

TABLE 5.—Side effects from BCG immunotherapy of prostatic carcinoma

Patient No.	Fever	Treatment for fever*	Hypotension	Malaise	Therapeutic response
1	Yes	ASA, INH	No	Yes	Good
2	Yes	ASA, INH	No	Yes	Good
3	Yes	ASA, INH	No	Yes	Good
4	Yes	Benadryl, steroids, INH	Yes	Yes	Good
5	Yes	ASA, INH	No	No	Good
6	Yes	ASA, INH	No	No	Good
7	Yes	Benadryl, steroids, INH	Yes	Yes	Good

*ASA = aspirin; INH = hydrazide of isonicotinic acid.

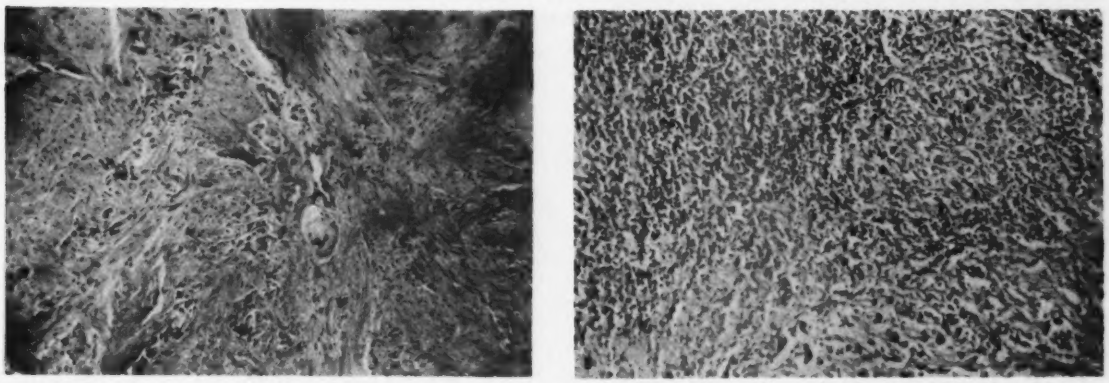

FIGURE 1.—Patient No. 3: carcinoma of the prostate. (*A*) Before injection with BCG. (*B*) Necrosis of the tumor after injection with BCG.

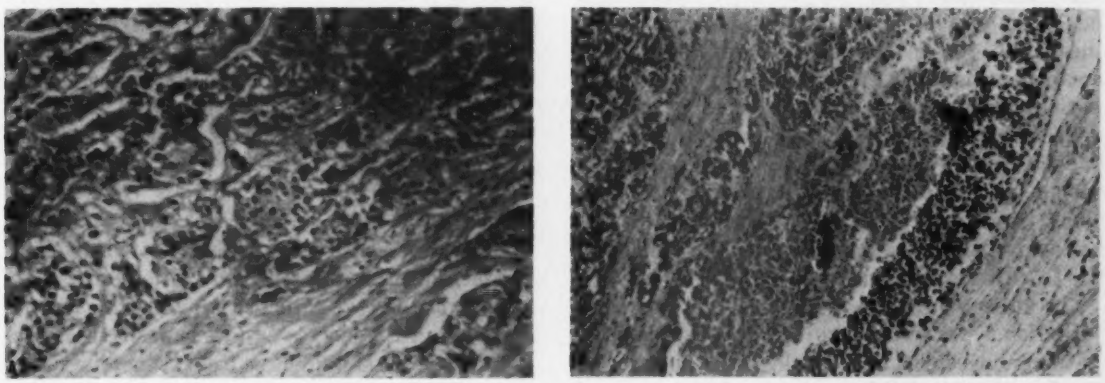

FIGURE 2.—Patient No. 6: carcinoma of the prostate. (*A*) Before injection with BCG. (*B*) Necrosis of the tumor after injection with BCG.

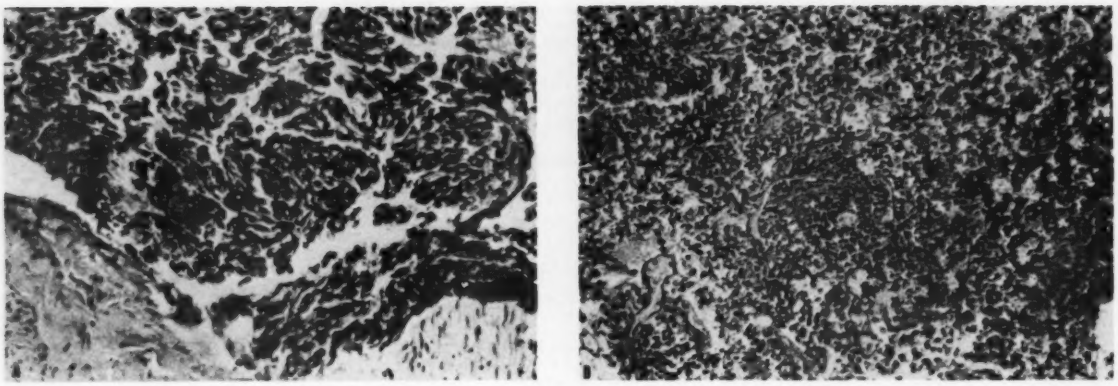

FIGURE 3.—Patient No. 2: carcinoma of the prostate. (*A*) Before injection with BCG. (*B*) Necrosis of the tumor after injection with BCG.

reactivity. This correlates very well with the immunodepression which exists in other advanced tumors like breast, lung (13), and bladder (14). In these tumors, the clinical staging is in direct relation to the degree of immunodepression. This also seems to be true in carcinoma of the prostate.

One third of our patients showed abnormalities in their protein electrophoresis with an increase of the β-globulin fraction. Immunoglobulin levels were altered in the same number of patients and an increase in IgM immunoglobulins was noted. Gursel et al (15) made the same observation in a series of 16 patients with carcinoma of the prostate. The meaning of these alterations has not yet been established, but a superficial explanation could attribute these findings to the presence of circulating antibodies against the tumor. The number of patients who responded to BCG therapy is very surprising considering the advanced stage of the tumor in this type of patient. The response was local only (at the site of injection), and the lack of response of distant metastases could be explained by the failure of the BCG to reach them.

An interesting observation was the lack of granuloma formation in the prostate of the patients injected. That could be explained by the fact that the BCG was injected in an area of the body where it could not find propitious surroundings. I suspect that BCG, like the mycobacterium tuberculosis, has affinity for organs with a high concentration of oxygen. This would explain the migration to other organs and the common formation of granulomas in the lungs.

The action of BCG on tumors (16) has been described as a two-stage response: first, an initial reaction specific to the BCG, and second, an antitumor action which is less understood and is nonspecific. For the first phase to occur, immunologic competence is required (as expressed by positive PPD skin test). For the second phase to occur, it is necessary for the BCG to enter in contact with the tumor.

Mitchell et al (16) showed that lymphocyte activity is mediated by macrophages which are first stimulated and produce a lymphocyte-activating factor which transforms the lymphocyte into a killer cell against the tumor. In the case of BCG stimulation, it seems that the mechanism is different. The lymphocyte is not acting as a killer cell. The destruction of the tumor (17–20) is performed by cells of the macrophage histiocyte series. This would explain the absence of lymphocytes in biopsies taken from the areas of BCG injection.

In our study we failed to convert the patients who were PPD negative to PPD positive. Retrospectively, we should have used transfer factors from PPD-positive patients instead of trying to vaccinate these patients with BCG.

In conclusion, nonspecific immunotherapy of

prostatic carcinoma with BCG is capable of producing an objective regression of the tumor at the site of the injection. This action alone does not appear to be enough to control the growth of the tumor. But, in conjunction with other therapeutic measures, immunostimulation with BCG may improve the prognosis in these patients.

In the future, patients with an earlier disease stage and a smaller tumor may prove to be more favorable subjects for the clinical investigation of this form of therapy.

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Extended-Field Radiation Therapy for Carcinoma of the Prostate: A Progress Report [1]

Malcolm A. Bagshaw, Gordon R. Ray, John R. Salzman, and Edwin M. Meares, Jr.[2,3]

Before discussing the rationale for extended-field radiation therapy in carcinoma of the prostate, it is of interest to review briefly the historical role of radiotherapy in prostatic cancer and some of the developments which have stimulated this broader approach to its therapy. Radiation therapy was first employed by means of an intraurethral radium source by Pasteau in 1911 (1) and Pasteau and Degrais in 1913 (2). This method and modifications thereof were later adopted in this country by Young et al (3), Barringer (4), and Deming (5). For example, in 1942 Barringer reported two instances of total sterilization of the prostatic region by radium needle implants (6). In 1934, Widman noted dramatic relief of obstructive symptoms, both ureteral and urethral, in patients with advanced prostatic cancer following kilovoltage radiotherapy to large pelvic radiation volumes (7). Because they were treating advanced disease, it is likely that many of their patients had responsive lymph node metastases. Renewed interest in direct interstitial irradiation of prostatic neoplasms was stimulated by Flocks et al (8) who noted regression of both the primary tumor and adenopathy after the direct injection of a suspension of radioactive gold colloid. More recently, Hilaris et al (9) introduced a method of interstitial irradiation by implantation in the prostate of ^{125}I seeds. The use of megavoltage radiation was first reported by Budhraja and Anderson (10) in a small series of patients treated with megavoltage teletherapy.

The patients at Stanford University School of Medicine were first treated in 1956 with definitive external-beam radiation therapy. Target volumes closely tailored to the prostatic region were irradiated with 4.8-MeV X-rays produced by the Stanford medical linear accelerator or by the 4- or 6-MeV Varian Clinacs, to doses in the range of 7000-8000 rads delivered at the rate of 1000 rads/week. In the 16 years between 1956 and December 1972, 405 patients were treated (11,12). It was possible to divide these patients into two general categories: (*a*) those with neoplasm confined to the prostate (similar to the stage B of the Veterans' Administration Cooperative Urological Research Group [VACURG]); and (*b*) those with the tumor extending through the capsule into either the seminal vesicles or other periprostatic tissues (similar to the VACURG stage C). Figure 1 demonstrates that when the tumor is confined within the capsule, 72% survival may be achieved at 5 years and 44% at 10 years. Although the survival is significantly decreased in patients with extracapsular extension, 51% survival was achieved at 5 years and 38% at 10 years. This significant difference between the survival of patients with extracapsular involvement and those with intracapsular disease prompted us to examine more closely the role lymph node metastases might play in this difference. Although lymphadenopathy has been emphasized in the surgical literature, especially by Flocks et al (13), Arduino et al (14), and Whitmore et al (15), it has been difficult to identify lymphadenopathy prior to radical re-

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[2] Division of Radiation Therapy, Department of Radiology (Drs. Bagshaw, Ray, and Salzman), and Division of Urology, Department of Surgery (Dr. Meares), Stanford University School of Medicine, Calif.

[3] *Reprint requests to:* Malcolm A. Bagshaw, MD, Division of Radiation Therapy, Department of Radiology, Stanford University School of Medicine, Stanford, Calif 94305.

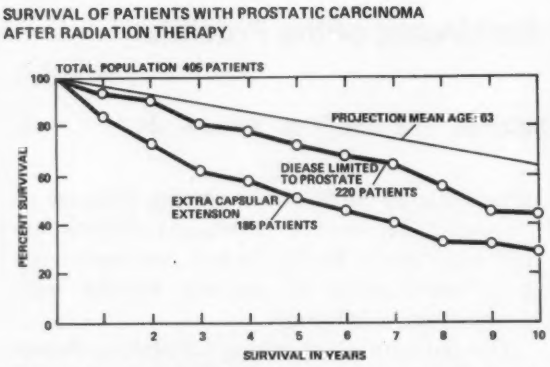

FIGURE 1.—Survival of patients with prostatic carcinoma after radiation therapy.

section. Following radical prostatectomy, lymph node sampling, and/or dissection, pathologic examination of the specimens has indicated that extracapsular involvement, and especially involvement of the seminal vesicles, is correlated with lymphadenopathy.

It is the purpose of this study to determine (*a*) the validity of lymphangiography in the detection of lymph node metastases; (*b*) the temporal relationship between lymph node metastases and osseous metastases; (*c*) the potential of radiation therapy in the sterilization of both overt and occult adenopathy; and (*d*) whether or not the increased burden of extended-field radiation therapy induces more adverse sequelae than can be justified by a potential increase in cure.

EVALUATION OF PATIENTS

Patients with apparently localized primary carcinoma of the prostate, 70 years of age or less, are thoroughly evaluated prior to a staging laparotomy. In addition to ordinary laboratory tests, these studies include serum phosphatase determinations, iliac crest Jamshidi bone biopsy, bone marrow acid phosphatase, metastatic survey, intravenous pyelography, lymphangiography, and technetium 99m-labeled-diphosphonate bone scans. Depending upon these findings, as illustrated in table 1, a decision is made regarding laparotomy. In essence, no laparotomy is carried out if the bone marrow biopsy is positive, the metastatic bone survey is positive, or the bone scan is clearly positive. Positive or negative serum phosphatase studies, bone marrow acid phosphatase levels, intravenous pyelograms, or lymphangiograms do not disqualify the patient for laparotomy. An important feature of the protocol is the serious attempt to include all patients who have no medical contraindication to surgery. We are

TABLE 1.—Prostate protocol: evaluation of patients with localized primary tumor, ≤ 70 years of age

Diagnostic studies	Results	Staging laparotomy
Serum	+	Yes
	−	Yes
Bone marrow biopsy	+	No
	−	Yes
Bone marrow acid phosphatase	+	Yes
	−	Yes
Metastatic survey	+	No
	−	Yes
Intravenous pyelography	+	Yes
	−	Yes
Lymphangiography	+	Yes
	−	Yes
Bone scan	+	No
	−	Yes

cognizant of the dangers of bias due to patient selection and believe that one way to minimize this bias is to maintain, insofar as possible, a consecutive series.

STAGING LAPAROTOMY

A transperitoneal laparotomy keyed by the lymphangiogram has been the procedure employed to date. Suspicious lymph nodes are excised. In the event of a negative lymphangiogram, randomly selected lymph nodes are removed from the iliac and the para-aortic node clusters. As a matter of routine, the obturator nodes have not been sampled and there has been no concerted attempt to carry out a complete node dissection. In some instances, however, fairly complete node dissections have been performed. The effort is to balance a minimal surgical procedure against the necessity to obtain a reasonable confirmation of the lymphangiographic findings.

At the time of laparotomy, the surgeon sketches the aorta on one of the lymphangiogram films and notes the position of all nodes removed. These are identified and submitted for pathologic examination. Multiple sections are cut from each node and evaluated for metastases. The position of the involved nodes is identified on a diagrammatic sketch, and a standardized summary sheet which includes pertinent clinical and laboratory data is prepared. Then the radiotherapist, surgeon, and pathologist convene a staging conference in order to establish by mutual agreement the clinical stage of the primary lesion and the confirmation of this by the surgical and pathologic findings. The staging system is indicated in the appendix. This system is an elaboration of the TNM system introduced by the Union Internationale Contre le Cancer (UICC). Recently, the prostate staging subcommittees of the UICC and the American Joint Committee have met and have reached an agreement on a new staging system based on the TNM concept.[4] Although the new TNM system has not been published, it has many points in common with the staging system presented here. When the new system becomes available, we will probably adopt it for this study.

[4] Rubin P. Personal communication, 1973.

RESULTS

To date, 34 patients have been studied. Clearly, the followup period is too short to allow a meaningful comment on survival; however, certain trends concerning diagnostic and prognostic parameters appear to be emerging.

Lymphangiography

The lymphangiogram was interpreted correctly in 15 of 18 patients with stage T_0, T_1, or T_2 (fig 2). There were, however, two false-positive interpretations and one false-negative. Eleven of 16 T_3 and T_4 patients had surgically proven adenopathy. The lymphangiogram correctly identified six of the 11, but failed to detect metastases in five. There was only one false-positive among the 16 patients with T_3 and T_4 involvement. Clearly, lymphangiography cannot be expected to detect microscopic or minimal foci, and a negative lymphangiogram alone is insufficient to rule out the substantial probability that lymph node metastases may be present in stages T_3 and T_4. Other factors to be discussed later may permit the identification of a subgroup of patients in which lymph node metastasis is highly unlikely.

Acid Phosphatase Determination

A correlation between the stage of the primary neoplasm, the serum acid phosphatase determination, and positive lymph nodes is presented in table 2. Note that there is a relatively large number of patients with apparently elevated acid phosphatase levels who have normal lymph nodes, particularly in the early stages. For example, for primary stages T_0, T_1, and T_2, 12 patients had an elevated acid phosphatase level whereas only two of these patients had positive lymph nodes. For the more advanced primary stages (that is, T_3 and T_4), ten patients had elevated acid phosphatase levels while eight of these had adenopathy. Reference to table 3, however, shows that of the 18 patients with elevated acid phosphatase levels prior to therapy, 15 converted to normal following radiation therapy. Of these 15, 14 have no evidence of disease and one has soft

FIGURE 2.—A lymphangiogram demonstrating a filling defect in an enlarged left external iliac lymph node. Most of the node is replaced by neoplasm and, therefore, does not fill with the contrast agent. The peripheral sinus of the inferior and lateral border of the node is opacified, producing the typical "egg shell" sign.

tissue metastasis. The nine patients with normal acid phosphatase levels prior to treatment remained normal after treatment, although one of these died with evidence of disease. These data are inconclusive and raise questions concerning the validity of the normal range as established in our laboratory. We have recently introduced the sodium thymophthalein monophosphate test. The question of a suitable methodology for acid phosphatase determination will be the subject of a more detailed analysis.

BONE METASTASES

None of the patients in this series had evidence of bone metastases by roentgen skeletal survey. Similarly, all of the Jamshidi iliac crest bone biopsies have been negative. Several focal areas of increased technetium 99m-labeled-diphosphonate have been detected. These have been at bizarre sites, such as the ankle or the knee, and in the face of negative roentgen studies have been interpreted as insufficient evidence for osseous metastases. Periodic bone scans will be obtained on these patients so that the validity of this interpretation may be evaluated in the future.

TREATMENT

Eleven patients have been randomized to prostatic irradiation only, 16 to prostatic irradiation plus pelvic adenopathy, and seven to prostatic irradiation plus pelvic irradiation plus para-aortic node irradiation. The radiation volumes are illustrated in figure 3 (*A* and *B*).

Nothing can be said at this time concerning relative survival. Only one patient has died and his death was due to a myocardial infarct 11 months after therapy. He had a focus of carcinoma in the prostate and two microscopic foci in the lung. One other patient has known soft tissue metastases. The remaining patients are living without evidence of disease. It is possible to state, however, that the burden of the extended-field radiotherapy, at least in the patients treated early, has been severe. This has been manifested by a sharp increase in radiation enteritis confined to the small bowel. This sequela was not seen in our previous series of 405 patients treated to the prostatic region only because the small-field technique confines the radiation to the prostatic region inferior to the abdominal cavity. The same is true of the 11 patients in this series who have received local irradiation only, none of whom has significant radiation sequelae. Among the 16 patients who received irradiation to the pelvis as well as the prostate, four developed symptoms of small bowel obstruction, with three responding to

TABLE 2.—Relationship between primary stage, serum acid phosphatase level, and lymphadenopathy*

Stage	Normal acid phosphatase level			Elevated acid phosphatase level		
	Total No. of patients	No. with negative nodes	No. with positive nodes	Total No. of patients	No. with negative nodes	No. with positive nodes
T_0	0	0	0	1	0	1
T_1	2	2	0	3	3	0
T_2	4	4	0	8	7	1
Totals	6	6	0	12	10	2
T_3	6	3	3	9	2	7
T_4	0	0	0	1	0	1
Totals	6	3	3	10	2	8

* In our laboratory the normal range is 0.13–0.64 Bessie Lowry units for total acid phosphatase and 0.01–0.15 Bessie Lowry units for tartrate inhibitable prostatic fraction.

TABLE 3.—Serial determinations of acid phosphatase in 27 patients (followup, 1–26 months)

Acid phosphatase levels at—		
First pretreatment examination	Last followup visit	Clinical status
Abnormal, 18 patients	Abnormal, 3 patients	No evident disease, 3 patients (11–16 mos)
	Normal, 15 patients	No evident disease, 14 patients (1–25 mos)
		Soft tissue metastasis, 1 patient
Normal, 9 patients	Normal, 9 patients	No evident disease, 8 patients (1–26 mos)
		Died with disease, 1 patient

conservative management and one requiring resection of a damaged loop of the small bowel. When para-aortic irradiation was added to total pelvic irradiation in the remaining seven patients, six had symptomatic small bowel disease and four required resection. The symptoms of small bowel dysfunction occurred 5–25 months after therapy. Fortunately, the small bowel resections have been successful in all instances. It is of interest that in three of the four patients who were treated to the para-aortic region because of proven para-aortic node metastases and who later required small bowel resection, no evidence of persistent neoplasm could be found by a careful search of the para-aortic region.

This sharp increase in small bowel reactions is of considerable concern. There are several plausible explanations, any one or any combination of which could increase radiation damage to the small bowel. Firstly, it has been well documented in other situations in which laparotomy precedes radiation therapy that the incidence of small bowel damage is increased, presumably due to decreased bowel mobility. This has been demonstrated in our own past experience with patients with cervical carcinoma. Secondly, the total dose (that is, 5500 rads delivered to such a large abdominal volume in patients in this age group) may be too high. And finally, in the first group of patients, radiation was delivered through only two fields each day, to each volume under treatment. From the standpoint of technique, this is less desirable than delivering the radiation through all fields each day in order to achieve the

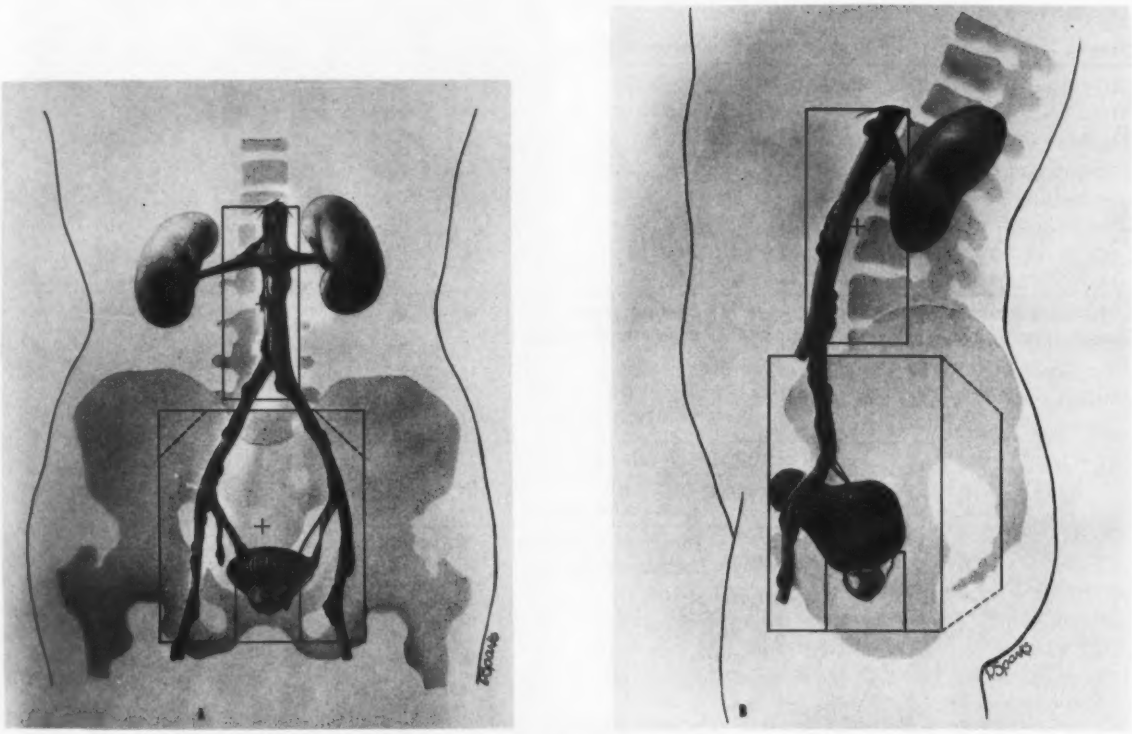

FIGURE 3.—Radiation portal arrangement for treatment of the prostate, pelvic lymph nodes, and para-aortic nodes. A 4-field arrangement is used for the treatment of the pelvis to 2500 rads in 2½ weeks at which time the field is reduced and re-centered over the prostatic region. An additional 2000 rads in 2 weeks is delivered to the prostate, after which the field is returned to its original full pelvic configuration and an additional 2500 rads in 2½ weeks is delivered. The para-aortic region is treated with anterior and posterior opposed fields and left and right slightly angled lateral fields (to avoid the kidneys) to 2500 rads at the rate of 1000 rads/week. Patient tolerance permitting, this region may be treated both before and after the prostatic boost concomitantly with the treatment of the large pelvic fields. Thus, at the conclusion of the prostatic boost, the second half of the para-aortic treatment (an additional 2500 rads in 2½ weeks) is administered. This permits a "split-course" approach to the large pelvic and para-aortic regions to a total dose of 5000 rads in 7 weeks, while the prostate receives 7000 rads in 7 weeks.

maximum homogeneity of radiation in the treatment volume. Incidentally, this requires treating as many as eight fields per day, a substantial but acceptable technical burden. We have subsequently modified the radiation technique by (*a*) reducing the dose to the para-aortic region from 5500 to 5000 rads; (*b*) treating all fields each day; and (*c*) introducing a modified split-course technique for the pelvic region. The modification consists of delivering 2500 rads to the entire pelvis in a period of 2½ weeks, followed by a 2000-rad boost delivered to the prostate region in only 2 weeks, and then completing the pelvic treatment with an additional 2500 rads in 2½ weeks (table 4). In effect, this lengthens the time of irradiation from 5 to 7 weeks for the small bowel (which is unavoidably adjacent to potential metastases in lymph nodes), while at the same time permitting continuous therapy to the prostatic region to a dose level of 7000 rads in 7 weeks. Since these modifications were adopted, only one patient has had mild enteritis which has been treated by conservative means. If radiation

TABLE 4.—Stanford University treatment schedules

Presumed extent of disease	Site of treatment	Dose (rads)	Total dose (rads)
Prostate + pelvic nodes	Pelvis (4 fields)	2500 in 2½ wks	
	Prostate (rotation)	Boost of 2000 in 2 wks	5000 in 7 wks (pelvic nodes)
	Pelvis (4 fields)	2500 in 2½ wks	7000 in 7 wks (prostate)
Prostate + pelvic nodes + para-aortic nodes	Pelvis (4 fields) + Para-aortic nodes (4 fields)	2500 in 2½ wks 2500 in 2½ wks	
	Prostate (rotation)	2000 in 2 wks	5000 in 7 wks, split course (pelvic + para-aortic nodes)
	Pelvis (4 fields) + Para-aortic nodes (4 fields)	2500 in 2 wks 2500 in 2 wks	7000 in 7 wks (prostate)

enteritis continues to be a problem, we would consider modifying the surgical approach to a retroperitoneal exploration, such as is employed by others. We have not employed the retroperitoneal approach thus far because of our desire to achieve adequate sampling of the para-aortic lymph nodes.

Although more data will be required to establish criteria for the selection of patients for lymph node sampling, the study to date has demonstrated some interesting trends. For example, of six T_1 and T_2 patients who had normal lymphangiograms and normal acid phosphatase levels, none has had positive lymph nodes. The single T_0 patient did have positive lymph nodes, but this patient also had an elevated acid phosphatase level. Of nine T_3 and T_4 patients with lymphangiograms positive for metastatic disease and abnormal acid phosphatase levels, the abnormal lymphangiogram in each instance was confirmed by surgical staging. Thus, although these numbers are small, the trend indicates that these parameters might be extremely useful in determining which patients should receive prostatic irradiation only and which should receive extended-field irradiation. If these trends become firm, then laparotomy for the assessment of lymph node metastases will be unnecessary for patients who exhibit these parameters.

APPENDIX 1.—Current clinical staging system for prostatic cancer: May 27, 1974 (revised July 15, 1974)

The following is a proposed staging system to be adopted by the Division of Radiation Therapy, Stanford University School of Medicine, for both protocol and nonprotocol patients with prostatic carcinoma. It is an extreme modification of the UICC system. This staging system should be viewed as an interim one which facilitates more accurate recording of the extent of disease at the time the patient is first seen, and therefore will help to provide information upon which a clinically useful staging system may be developed in the future.

T: Primary Tumor

T_x = At the time of physical examination the characteristic anatomic relationships are distorted and/or absent secondary to major surgical intervention, ie, radical prostatectomy.

T_0 = Occult carcinoma: the incidental finding of carcinoma in the operative specimen.

T_1 = Palpable tumor limited by the prostatic capsule without distortion of the superior or lateral anatomic boundaries.

a. Solitary nodule $\leq$ 1 cm in diameter with normal, compressible, prostatic tissue on three sides (lesion amenable to radical prostatectomy).

b. Palpable tumor > 1 cm occupying < 50% of a lobe.

c. Palpable tumor occupying > 50% of a lobe, multiple nodules limited to one lobe, or involvement of both lobes.

T_3 = Palpable tumor of any size primarily limited by the prostatic capsule with minimal distortion of the lateral or superior anatomic boundaries without definite obliteration of a lateral sulcus and/or a seminal vesicle region.

a. Palpable tumor occupying < 50% of a lobe.

b. Palpable tumor occupying > 50% of a lobe, multiple nodules limited to one lobe, or involvement of both lobes.

T_4 = Palpable tumor extending beyond the prostatic capsule with definite obliteration of the lateral sulci and/or a seminal vesicle region.

a. Tumor involving < 50% of a lobe.

b. Palpable tumor occupying > 50% of a lobe, multiple nodules limited to one lobe, or involvement of both lobes.

T_4 = Palpable tumor extending beyond the prostatic capsule with attachment to both pelvic sidewalls or rectal wall, or bladder invasion.*

N: Nodes

Nodal designations will involve two subscripts (eg, N_{1A}). The first subscript designates the clinical impression, the second designates the biopsy status.

Clinical

X = Lymphangiogram (LAG) not performed or technically inadequate.

0 = LAG not consistent with carcinomatous involvement of the regional nodes; para-aortic nodes uninvolved.

1 = LAG consistent with carcinomatous involvement of the regional nodes; para-aortic nodes uninvolved.

2 = LAG consistent with carcinomatous involvement of the para-aortic nodes; regional nodes uninvolved.

3 = LAG consistent with carcinomatous involvement of the regional and para-aortic nodes.

4 = Pathologic adenopathy other than regional or para-aortic nodes, as demonstrated by lymphangiography, chest roentgenogram, or physical examination; eg, supraclavicular, mediastinal. (If biopsy is performed and is negative, disregard this category.)

Biopsy

X = Biopsy not performed or technically inadequate.

A = Regional and para-aortic nodes show no evidence of tumor.

B = Regional nodes positive, para-aortic nodes negative for tumor.

C = Regional nodes negative, para-aortic nodes positive for tumor.

D = Regional and para-aortic nodes positive for tumor.

E = Any of the above plus positive biopsy of nodal site outside regional or para-aortic nodes; if not biopsied, use ×.

M: Distant Metastases (excluding lymph nodes)

M_0 = No evidence of metastases.

M_1 = Isolated area of abnormal epithelial cells in bone marrow unassociated with multiple bony, soft tissue, or visceral metastases.

M_2 = Bony, visceral, or soft tissue metastases with or without bone marrow involvement.

Example

Patient with 25% of one lobe involved with tumor but invading right seminal vesicle whose LAG shows normal regional and para-aortic nodes. Biopsy reveals tumor in the regional nodes and negative para-aortic nodes; no evidence of metastases: stage $T_3N_{0B}M_0$.

*Patients with intravenous pyelogram findings suggestive of bladder involvement will undergo cystoscopy and biopsy if indicated.

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Potential Test Systems For Drugs Against Prostatic Cancer[1,2]

A. A. Sandberg, R. Y. Kirdani, H. Yamanaka, M. J. Varkarakis, and G. P. Murphy[3,4,5]

SUMMARY

A number of chemotherapeutic agents have been tested in two systems which could be useful as models in the search for effective drugs for cancer of the prostate. One system involved the effects of the administered drugs on rat prostatic 5α-reductase and arginase activities. Since both enzymic systems are androgen dependent and essential for prostatic function and anatomy, the effectiveness of a drug in these systems could be indicative of its value in the treatment of prostatic cancer. Michaelis constants were obtained with Lineweaver-Burk plots and the conclusions are based on a comparison of these plots with those of the controls. Thus, the following results were obtained: (*a*) isophosphamide, bleomycin, and procarbazine produced definite inhibition of 5α-reductase in either or both the ventral and dorsolateral glands of the rat; (*b*) 5-fluorouracil, vincristine, bleomycin, procarbazine, adriamycin, and hexamethylmelamine inhibited arginase activity significantly in both glands; (*c*) streptozotocin and 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (NSC-45388) produced inhibition of arginase in the ventral gland only; (*d*) in contrast to the noncompetitive or uncompetitive inhibition of most of the drugs, particularly in the ventral gland, procarbazine, hexamethylmelamine, bleomycin, adriamycin, and NSC-45388 produced competitive inhibition of arginase in the dorsolateral gland; and (*e*) seven of the drugs led to an activation of 5α-reductase (5-fluorouracil, vincristine, NSC-45388, hexamethylmelamine, CCNU, streptozotocin, and diglycolaldehyde). The second model system utilized the deposition of labeled estriol and testosterone in the dog prostate and the effects of drug therapy as a possible index of effectiveness in prostatic cancer. Streptozotocin and procarbazine definitely interfered with the deposition of both estriol and testosterone. On the basis of the data obtained, the model systems investigated by us could potentially serve as reliable indicators for the clinical use of drugs against cancer of the prostate.

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[1] Supported in part by Public Health Service grant CA-15436 from the National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[2] Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are listed in the List of Compounds at the end of the text. Adriamycin, CCNU, hexamethylmelamine, isophosphamide, NSC-45388, and streptozotocin were supplied by Cancer Therapy Evaluation, Division of Cancer Treatment, NCI.

[3] Roswell Park Memorial Institute, Buffalo, NY.

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[5] *Reprint requests to*: A. A. Sandberg, Roswell Park Memorial Institute, 666 Elm St, Buffalo, NY 14203.

The application and use of model systems for therapeutic agents against human cancers have usually depended upon the effect of such agents in animal tumors which are similar to those in man in their anatomic location, histologic picture, and biologic behavior. Or, they have also depended upon the development of drugs which would directly affect or interfere with a unique metabolic, secretory, or enzymatic system characteristic for a particular tissue, and thus these drugs would hopefully serve as antimetabolites with the ability to reverse the growth of tumors in these tissues.

Unfortunately, no suitable animal tumor model has been developed for cancer of the prostate. Therefore, in approaching the development of systems for the evaluation of drugs against human prostatic cancer, we first addressed ourselves to the testing of those agents which may affect two crucial and unique metabolic systems in prostatic tissue: (*a*) 5α-reductase (5α-RA) and arginase activities in the rat prostate; and (*b*) deposition of estrogens and androgens in the prostate of the dog. We would like to explain the rationale for selecting these systems.

It has been established that the 5α-RA system in the prostate of almost all animals tested is responsible for the conversion of testosterone (T) to dihydrotestosterone (DHT) and that the latter steroid appears to be primarily responsible for the androgenic effects so necessary for the maintenance of prostatic function and anatomy (1,2). Interestingly, the level of 5α-RA is preponderantly under the control of T, or DHT, or both, ie, the administration of T or DHT to castrated animals will not only restore prostatic weight to normal but will also elevate the 5α-RA to normal or above normal levels (3–5). The administration of T or DHT to intact rats may raise the 5α-RA above control levels.

On the other hand, the administration of estrogens to intact animals results in greatly decreased 5α-RA, and this has been adduced primarily to be due to interference with the luteinizing hormone release from the pituitary and consequent decreased secretion of T by the testes (1,6–8). The low levels of circulating T are then reflected in the decreased 5α-RA. However, there are those who believe that estrogens may have a direct effect on prostatic 5α-RA (9–11), an effect not requiring the intercession of pituitary or testicular effects.

Because of the efficacy of estrogens in remarkably alleviating the subjective and/or objective aspects of human prostatic cancer, and the key role played by 5α-RA in prostatic physiology, we felt that any drug affecting this enzymatic activity to a significant extent should be considered as a possible candidate for therapy for prostatic cancer.

The role of prostatic arginase is not as clearly understood as that of 5α-RA. The function of this enzyme is the deamination of arginase to ornithine (12–14) and, thus, it may play an important role in the synthesis of some polyamines (putrescine, spermidine, spermine) which are unique to the prostate and which constitute an important moiety of the secretory element of this gland (15–17). Similar to 5α-RA, arginase activity is largely under the control of androgens, although the activity of this enzyme after castration does not decrease to the same low levels that are observed for 5α-RA (12–14). Therefore, it is probable that substances other than androgens also control the level of arginase in the prostate, or that androgens control that moiety of the enzyme which is localized in specific prostatic cells (eg, epithelial cells) which are under some direct control of these steroid hormones vs the lack of control in other cells (eg, supporting cells). Our choice for testing the effects of drugs on arginase activity was based on the above considerations.

The second system used to test agents which are possibly useful in the treatment of prostatic cancer is based on the demonstration that the prostate of the dog, as well as that of the rat, baboon, and man, concentrates both androgens (T, DHT) and estrogens (estradiol-17β, estriol) (18–20). The presence of specific receptor proteins for these steroids has been demonstrated in prostatic tissue in the rat (21) with the same characteristics as those of other receptor proteins in other target organs, eg, uterus, breast, etc. The presence of such receptor proteins in the prostate for both androgens and estrogens

led us to believe that agents which interfered with the deposition of either or both steroid hormones in the prostate of the dog may be useful in the therapy of human prostatic cancer. This was based on the theory that steroids associated with receptor proteins play a key role in the transcription of the DNA in such cells and are therefore important regulators of cellular function.

In this paper we wish to present the results utilizing 12 chemotherapeutic agents in the two systems we have discussed.

MATERIALS AND METHODS

Male adult Wistar rats (weighing 330–450 g) purchased from Charles River Breeding Laboratories, Wilmington, Mass, were acclimated in our laboratory for about 1 week and injected with various drugs. Each drug was injected intraperitoneally daily for 1 week, except for diglycolaldehyde which was given for 5 days. On Day 8 the rats were killed by ether anesthesia and the ventral and dorsolateral prostates were removed.

5α-RA and arginase activities were determined by methods previously described (3,12, 25). The only modification was the introduction of tritiated DHT during incubation with ^{14}C-T to evaluate the possibility of further metabolic transformation of the DHT derived from the labeled T (5,26); the calculations of the 5α-RA took this factor into consideration. For the determination of the Michaelis constants, the concentration of T varied from 0.17 to 1.02 × 10^{-9} M, with at least five different concentrations being used for plotting the Lineweaver-Burk plots.

Adult male dogs were used for the deposition studies with labeled ^{3}H-estriol (E_3) and ^{14}C-T. The control group consisted of three dogs and each drug was given to two or three dogs. The methods for the administration of the labeled steroids and the determination of radioactivity in tissues have been described previously (18–20). The drugs were given for 4½ days, the last administration occurring on the day of the injection of the labeled E_3 and T, usually preceding this injection by 2–3 hours.

The drug doses given to the rats and dogs were established on the basis of comparable therapeutic doses in man and, in some cases, according to the effects observed in animal studies, particularly as described in the clinical brochures on the drugs supplied by the NCI.

Prior to injection, hexamethylmelamine (obtained in capsules) was dissolved in water; cyclophosphamide powder was dissolved in saline, CCNU tablets were crushed and dissolved in water, and procarbazine (obtained as a crystalline powder) was dissolved in water. All other drugs were administered in the solutions in which they were supplied either by the manufacturers or by the NCI.

For some drugs, the doses ultimately used had to be decreased due to the death of the animals caused by the drugs. In such instances, we usually decided upon a dose that was one third to one fifth of the lethal one.

Usually, three drugs were tested during each time period, with control animals accompanying each experimental group of rats. The control group consisted of ten rats and the drugs were administered to 18–30 animals per drug.

The ^{3}H-E_3 (6,7-^{3}H-E_3, 5 millicuries/μmol) and ^{14}C-T (4-^{14}C-T, 50 microcuries/μmol) were obtained from the New England Nuclear Corporation and their purity was checked by chromatography.

In deciding upon the length of time for the administration of drugs, it was felt that they should not be given for any period which would seriously affect total body weight or appetite, or would produce other untoward toxic effects in the animals.

The Michaelis constants (K_m and V_{max}) were established by Lineweaver-Burk reciprocal plots and the results expressed in terms of competitive, noncompetitive, or uncompetitive inhibition or activation (27). Competitive inhibition was thought to exist when the V_{max} did not change, while the apparent K_m showed a definite increase. Noncompetitive inhibition was thought to exist when there was a change in the V_{max} but no interference with the combination of the enzyme with its substrate. Uncompetitive inhibition was thought to exist when the slope of the linear plot essentially paralleled that of the control values. Activation took these same factors into consideration.

RESULTS

Each drug caused some decrease in body weight (table 1) during administration. The decreases ranged from 2% to 23% (mean, 13.75%) of the pretreatment weight, which compared with the 0–12% gain in body weight in the control rats.

It is interesting that on the average, the decrease in prostatic weight tended to be higher (and in the case of some drugs markedly so) than the decrease in body weight (table 1). The loss in prostatic weight, when the latter was compared to that of the control groups, ranged from 0 to 47% (mean, 19.5%) in the ventral gland and from 0 to 35% (mean, 16.5%) in the dorsolateral prostate. 5-FU, cyclophosphamide, isophosphamide, adriamycin, diglycolaldehyde, bleomycin, and CCNU caused the largest decreases in prostatic weight.

Effects on Rat Prostatic 5α-RA

The doses and routes of administration of the various drugs are given in table 2. Table 3 shows the K_m and V_{max} values for the 5α-RA in the ventral and dorsolateral prostates of the rat. These results have also been graphically represented in figures 1–4 as Lineweaver-Burk plots. We interpret the significance of our findings to be that isophosphamide, bleomycin, and procarbazine produce a definite inhibition of 5α-RA either noncompetitively or uncompetitively. This inhibition of the 5α-RA was manifested in the ventral and/or dorsolateral glands. On the other hand, 5-FU, vincristine, hexamethylmelamine, CCNU, streptozotocin, NSC-45388, and diglycolaldehyde actually produced either uncompetitive or noncompetitive activation of 5α-RA in either or both glands of the rat.

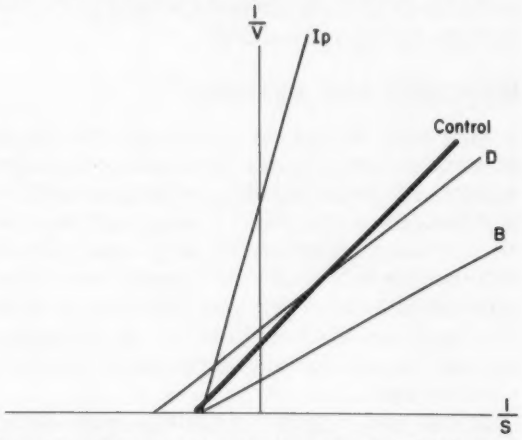

FIGURE 1.—Effects of isophosphamide (Ip), NSC-45388 (D), and bleomycin (B) on 5α-RA in the rat ventral prostate as shown by Lineweaver-Burk reciprocal plots and compared with control values' plot.

TABLE 1.—Effects of chemotherapeutic agents on rat body and prostatic weights

Drug	Decrease (%) in body weight compared to pretreatment weight	Prostatic weights (%) compared to controls*	
		Ventral	Dorsolateral
5-FU	−19	−26	−27
Cyclophosphamide	−23	−24	−20
Vincristine	−7	−1	−6
Procarbazine	−5	0	0
Hexamethylmelamine	−11	−16	−8
CCNU	−15	−28	−13
Isophosphamide	−22	−31	−18
Bleomycin	−23	−18	−35
NSC-45388	−2	−5	0
Streptozotocin	−13	−14	−20
Adriamycin	−10	−47	−31
Diglycolaldehyde	−15	−24	−17

*Control ventral prostatic weight: 402 ±23–485 ± 24 g. Control dorsolateral prostatic weight: 303 ± 15–344 ± 11 g.

TABLE 2.—Chemotherapeutic agents tested and dose administered to rats*

Drug	Dose (mg/kg/day)
5-FU	30
Cyclophosphamide	50
Vincristine	50†
Procarbazine	5
Hexamethylmelamine	50
CCNU	40
Isophosphamide	100
Bleomycin	5
NSC-45388	5
Streptozotocin	15
Adriamycin	2.5
Diglycolaldehyde	50

*Animals used were Wistar male rats (330–450 g). All drugs except diglycolaldehyde were injected intraperitoneally for 7 days; diglycolaldehyde was injected for 5 days. On Day 8 rats were killed under ether anesthesia and prostates were removed.

†μg/kg/day.

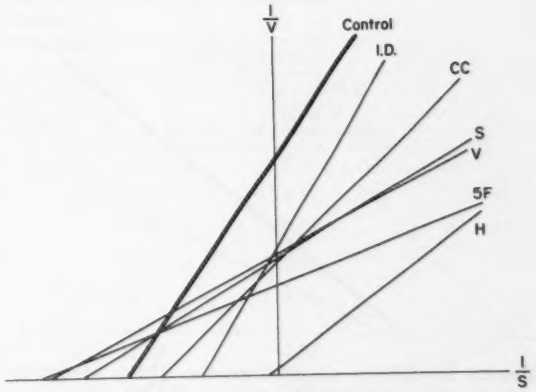

FIGURE 2.—Effects of diglycolaldehyde (ID), CCNU (CC), streptozotocin (S), vincristine (V), 5-FU (5F), and hexamethylmelamine (H) on 5α-RA in the rat ventral prostate as shown by Lineweaver-Burk reciprocal plots and compared with control values' plot.

TABLE 3.—Effects of chemotherapeutic drugs on 5α-RA of rat ventral and dorsolateral prostate

	Ventral		Dorsolateral	
Drug	$K_m \times 10^{-7}$ M	V_{max} (10^{-9} M/g of tissue/hr)	$K_m \times 10^{-7}$ M	V_{max} (10^{-9} M/g of tissue/hr)
5-FU*	0.36	2.13		
Cyclophosphamide	4.0	0.25		
Vincristine*	0.90	3.94		
Procarbazine	1.30	2.44	0.57	1.44
Hexamethylmelamine	1.43	0.65		
CCNU	1.73	4.17	0.95	4.72
Isophosphamide	1.56	0.62	0.25	0.67
Bleomycin	4.21	4.48	1.06	1.58
NSC-45388	0.84	1.57	1.75	4.06
Streptozotocin	1.04	4.02	3.76	8.27
Adriamycin	15.02	0.04	1.617	0.122
Diglycolaldehyde	2.56	5.49		
Controls	1.33	2.72	0.50	2.60

*Combined dorsolateral and ventral.

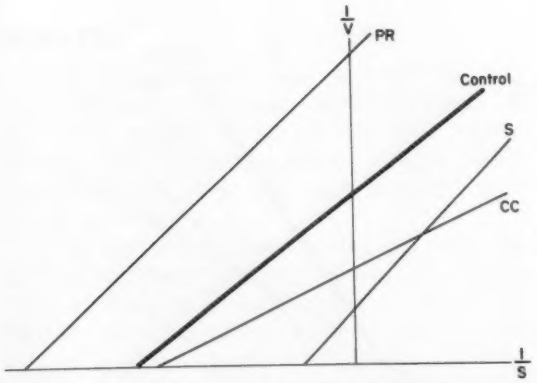

FIGURE 3.—Effects of procarbazine (PR), streptozotocin (S), and CCNU (CC) on 5α-RA in the rat dorsolateral prostate as shown by Lineweaver-Burk reciprocal plots and compared with control values' plot.

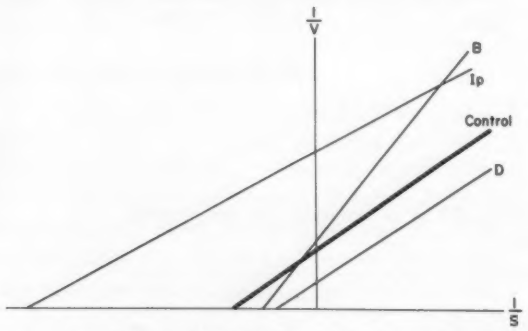

FIGURE 4.—Effects of isophosphamide (Ip), bleomycin (B), and NSC-45388 (D) on 5α-RA in the rat dorsolateral prostate as shown by Lineweaver-Burk reciprocal plots and compared with control values' plot.

Effects on Rat Prostatic Arginase Activity

The effects on arginase activity are shown in table 4 and figures 5 and 6. In contrast to the effects on 5α-RA, no activation of the enzymatic activity was observed with any of the drugs used. The results indicate that noncompetitive or competitive inhibition of arginase activity is demonstrated with vincristine, bleomycin, procarbazine, adriamycin, and hexamethylmelamine in both the ventral and dorsolateral prostate. On the other hand, streptozotocin and NSC-45388 produced noncompetitive inhibition only in the ventral gland. Bleomycin, hexamethylmelamine, procarbazine, and possibly adriamycin produced competitive inhibition only

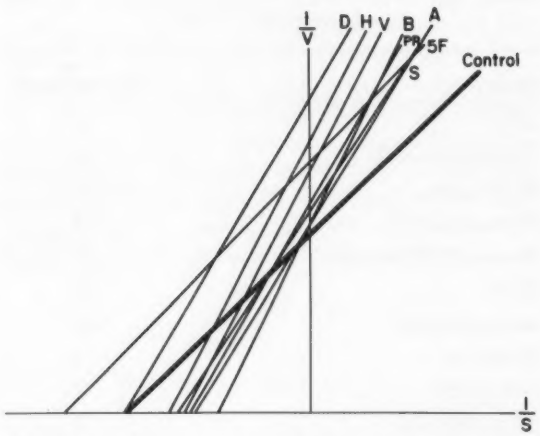

FIGURE 5.—Effects of NSC-45388 (D), hexamethylmelamine (H), vincristine (V), bleomycin (B), procarbazine (PR), adriamycin (A), 5-FU (5F), and streptozotocin (S) on arginase activity in the rat ventral prostate as shown by Lineweaver-Burk reciprocal plots and compared with control values' plot.

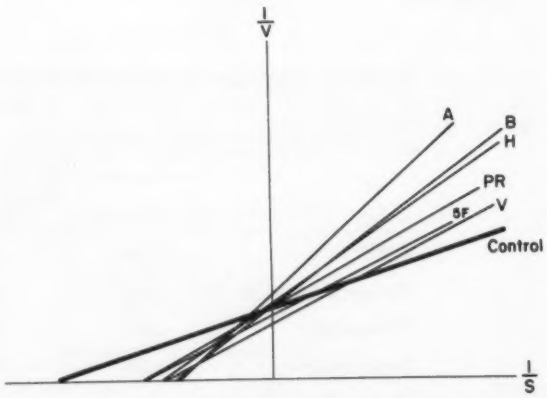

FIGURE 6.—Effects of adriamycin (A), bleomycin (B), hexamethylmelamine (H), procarbazine (PR), 5-FU (5F), and vincristine (V) on arginase activity in the rat dorsolateral prostate as shown by Lineweaver-Burk reciprocal plots and compared with control values' plot.

in the dorsolateral gland. It appears that 5-FU led to uncompetitive inhibition of arginase activity in both prostates. Cyclophosphamide, CCNU, isophosphamide, and diglycolaldehyde appeared to produce no effects in either prostate.

TABLE 4.—Effects of chemotherapeutic drugs on arginase of rat ventral and dorsolateral prostate

Drug	Ventral		Dorsolateral	
	$K_m \times 10^{-3}$ M	V_{max} (10^{-6} M urea/g of tissue/min)	$K_m \times 10^{-3}$ M	V_{max} (10^{-6} M urea/g of tissue/min)
5-FU	19.23	2.63	19.23	7.69
Cyclophosphamide	15.62	3.33	13.89	11.11
Vincristine	20.40	2.17	23.25	9.09
Procarbazine	21.28	2.50	18.52	6.67
Hexamethylmelamine	18.52	1.92	21.27	6.25
CCNU	10.00	2.50	11.62	6.25
Isophosphamide	11.76	3.33	11.11	6.25
Bleomycin	29.41	2.77	25.00	6.67
NSC-45388	13.89	1.67	11.49	5.56
Streptozotocin	10.53	2.08	13.34	6.25
Adriamycin	22.20	2.77	26.32	5.88
Diglycolaldehyde	18.50	3.23	12.66	6.67
Controls	13.33±0.63	2.89±0.15	11.50±0.41	6.72±0.43

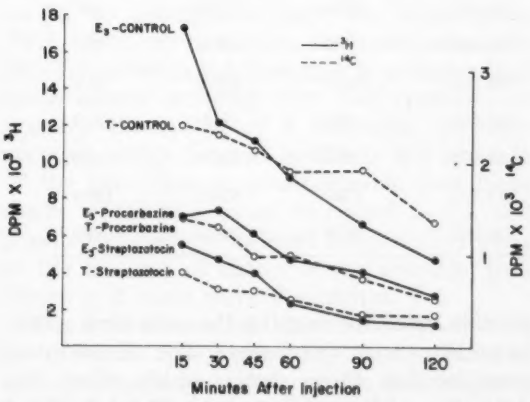

FIGURE 7.—Deposition of radioactivity in the dog prostate following the iv injection of labeled ^{3}H-E_3 (50 microcuries) and ^{14}C-T (10 microcuries). Both streptozotocin and procarbazine caused markedly decreased deposition of radioactivity in the gland.

TABLE 5.—Chemotherapeutic agents tested and dose administered to dogs*

Drug	Dose (mg/kg/day)	Route of administration
5-FU	15	Iv
Cyclophosphamide	20	Im‡
Vincristine	50†	Iv
Procarbazine	5	Iv
Hexamethylmelamine	50	Oral
CCNU	50	Oral
Isophosphamide	50	Iv
Bleomycin	1.5	Iv
NSC-45388	35	Iv
Streptozotocin	3	Iv
Adriamycin	2.5	Iv
Diglycolaldehyde	20	Iv

*Drugs given for 4 ½ days; last administration occurred on day of labeled E_3 and T injection.
†μg/kg/day.
‡Im = intramuscular.

Effects on the Deposition of Labeled E_3 and T

Table 5 shows the doses and routes of administration of the various drugs given to dogs. Table 6 and figure 7 present the results on the deposition of the intravenously (iv) injected pair of steroids, ^{3}H-E_3 (50 microcuries) and ^{14}C-T (10 microcuries). Based on our past experience, we interpret the results to be significant if there is a deviation of 40%–60% of the control values. The data indicate that pro-

carbazine and streptozotocin definitely interfere with the deposition of both E_3 and T. Hexamethylmelamine appears to cause a definite increase in E_3 deposition. The results in the testes and pancreas are shown for comparison, since deposition of E_3 in the latter organ (18) has been shown to be very extensive and changes in the deposition in the testes could affect prostatic parameters. None of the drugs inhibited the deposition of either E_3 or T in the pancreas; however, isophosphamide and bleomycin actually caused an increased E_3 concentration. Streptozotocin led to a definitely decreased deposition of E_3 and T in the testes, whereas cyclophosphamide caused an increase in T deposition.

TABLE 6.—Deposition of radioactivity in dog tissues following the iv injection of ^{3}H-E_3 and ^{14}C-T (^{3}H/^{14}C = 5/1)

	Deposition of radioactivity (dpm/g) in—					
	Prostate		Pancreas		Testes	
Drug	E_3	T	E_3	T	E_3	T
Control	10,100	1700	20,000	1200	2900	720
5-FU	15,300	3900	23,800	1800	2700	1200
Cyclophosphamide	16,200	2100	26,800	1700	4700	1400
Vincristine	11,600	2000	26,100	1400	3000	1200
Procarbazine	5400	950	22,800	1000	1800	500
Hexamethylmelamine	19,000	2000	23,900	1100	2600	700
CCNU	15,500	2400	24,000	1300	3200	900
Isophosphamide	14,000	1100	36,400	900	1800	600
Bleomycin	14,100	1800	33,600	1400	2900	800
NSC-45388	8600	1400	22,600	900	1800	800
Streptozotocin	3250	530	17,300	900	1200	430
Adriamycin	17,300	2800	33,700	2700	6700	1800
Diglycolaldehyde	10,700	1750	14,250	1500	2600	600

DISCUSSION

Even though the prostatic parameters, ie, 5α-RA and arginase activity in the rat and the deposition of E_3 and T in the dog, may not have any direct bearing on the treatment of prostatic cancer (particularly if the cancer does not contain substantial concentrations of the enzymes or the receptor proteins for steroid hormones), we feel that the approaches we utilized are worthy of application to the therapy of cancer of the prostate. It is possible that cancer of the prostate may have features which are comparable to some of those for cancer of the breast. In the latter, the presence of receptor proteins for estrogens appears to be a reliable index for varying the endocrine *milieu* in patients with the disease (28). Hence, it is possible that those drugs which affect the deposition of E_3 and T, possibly through direct effects on the receptor protein, may be useful in cancer of the prostate through their effects on hormonal conditions in the gland.

The effects on 5α-RA and arginase activity could be profitably transferred to the clinical condition if human prostatic cancer contained respectable concentrations of either or both enzymes. Although the incidence and levels of 5α-RA in a large series of such tumors have not been established, the presence of this enzyme has been definitely demonstrated in prostatic

cancer.[6] Arginase activity has been shown to be present in tissues from cancer of the prostate (13). Thus, it is possible that those drugs which affect both or either of the enzymes should be given a trial in the therapy for cancer of the prostate.

It is possible that some or all of the effects of some of the drugs administered may be mediated through the pituitary system and/or the testicular function. Thus, decreased T secretion by the testes as a result of a drug's effect on the luteinizing hormone release or synthesis may be reflected in decreased 5α-RA and arginase activities (as with bleomycin administration).

The demonstration of certain unusual effects of drugs on 5α-RA and arginase is of interest and deserves comment. The enhancement of 5α-RA by some agents has been reported by us previously (29), in which the results were based on a simple comparison of the percentage of conversion of T to DHT in prostatic tissue of normal rats vs that of animals who had received various drugs. In the present study, at least one half of the drugs used caused activation of 5α-RA as determined from the Lineweaver-Burk plots. Even though the mechanism for this activation is not known, it is probable that these agents interfere with the synthesis or possibly the action of a substance (or substances) which inhibits 5α-RA in the prostate of the rat. Thus, it is conceivable that these drugs would lead to an increased 5α-RA and possibly aggravate (at least temporarily) some of the features of cancer of the prostate, particularly if these were T sensitive.

The most intense inhibition of 5α-RA in both rat prostates was produced by isophosphamide, with bleomycin and procarbazine inducing such intense inhibition only in the dorsolateral gland. Adriamycin produced intense arginase inhibition in the latter prostate and 5-FU and NSC-45388 produced inhibition in the ventral gland.

The demonstration of competitive inhibition of arginase by several of these drugs is of interest, indicating an effect on the combination of the enzyme with its substrate. Such competitive inhibition was not apparently observed with 5α-RA; this is not surprising in view of the difference in the tertiary structures of the drugs administered as compared with those of the steroidal hormones.

Except for bleomycin and isophosphamide, those drugs which caused the most intense decreases in 5α-RA and arginase did not lead to the most prominent decreases in either body or prostatic weights. Furthermore, except for bleomycin and procarbazine, those drugs which caused a significant reduction in arginase activity did not necessarily cause a decrease in 5α-RA.

The parameters in this study were measured after a rather short period of drug administration (4.5–7 days), and it is possible that even more profound effects might be seen with some of the chemotherapeutic agents which were ineffective during short-term administration if they were given for longer time periods (weeks or months). Of course, the untoward effects which might result from prolonged drug administration may preclude such a study or affect their clinical efficacy, but we do plan to undertake such an approach.

LIST OF COMPOUNDS

Adriamycin: NSC-123127

Bleomycin: NSC-125066

CCNU: NSC-79037; CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan

Diglycolaldehyde: NSC-118994; diglycolaldehyde, α-(hydroxymethyl)-α′-(6-hydroxy-9*H*-purin-9-yl)-; inosine dialdehyde

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8; 5-FU

Hexamethylmelamine: NSC-13875; CAS reg. No. 645-05-6; HMM

Isophosphamide: NSC-109724; CAS reg. No. 3778-73-2; 1,3,2-oxazaphosphorine, 3-(2-chloroethyl)-2-[(2-chloroethyl)amino]tetrahydro-, 2-oxide

NSC-45388: CAS reg. No. 4342-03-2; 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide; DTIC

Procarbazine: NSC-77213; CAS reg. No. 491-88-3; *p*-toluamide, *N*-isopropyl-α-(2-methylhydrazino)-, monohydrochloride

Streptozotocin: NSC-85998

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

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New Model for Studying the Effects of Cancer Chemotherapeutic Agents on the Growth of the Prostate Gland [1,2]

William R. Sloan, W. D. W. Heston, and Donald S. Coffey [3,4]

SUMMARY

A new model for testing the ability of cancer chemotherapeutic agents to inhibit androgen-induced growth in the ventral prostate of castrated rats has been developed. This system involves the ability of drugs to inhibit the twentyfold stimulation observed in the rate of DNA synthesis in castrated rats receiving exogenous testosterone propionate for 3 days. It has been determined that a peak occurs in the rate of DNA synthesis 72 hours after an initial injection of testosterone propionate, and this has been used as an index to measure the ability of various cancer chemotherapeutic agents to inhibit this process. 5-Fluorouracil and adriamycin were effective drugs in this regard, and cyclophosphamide, CCNU, and streptozotocin were essentially without effectiveness on DNA synthesis. The merits of this model as a screen in determining the effects of these drugs on the growth of the prostate are discussed.

[Cancer Chemother Rep 59:185–194, 1975]

Presently, the treatment of human prostatic cancer encompasses three primary areas: surgery, chemotherapy, and radiation therapy. Since the early 1940s, surgical castration and/or estrogen administration have been used widely for the treatment of metastatic prostatic adenocarcinoma. For the most part, chemotherapy has been limited to hormonal treatment, including estrogens and, more recently, antiandrogens (1–4). The prevalence of prostatic cancer and the survival times of patients receiving these types of therapy indicate that these modalities may be primarily palliative, although in limited cases remarkable remissions have been observed. The lack of hormonal response in some patients coupled with the inevitable relapse which occurs in those patients responding to hormonal therapy have continued to stimulate endeavors for alternative chemotherapeutic approaches to the treatment of prostatic cancer. The evaluation of drugs which are effective against other types of cancer has been hampered by the reluctance of investigators to initiate clinical trials using cytotoxic agents in the treatment of human prostatic cancer, particularly in elderly patients where some benefit might be anticipated from the standard and accepted hormonal therapy. In addition, there is no adequate animal model for prostatic cancer suitable for the rational selection of nonhormonal drugs which could be effective in treating prostatic carcinoma. In

[1] Supported by Public Health Service grant CA-15416 from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

[2] Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are listed in the List of Compounds at the end of the text.

[3] The James Buchanan Brady Urological Institute of The Johns Hopkins Hospital (Dr. Sloan); Department of Pharmacology and Experimental Therapeutics, The Johns Hopkins University School of Medicine (Dr. Heston); and The Oncology Center, The Johns Hopkins Hospital (Dr. Coffey), Baltimore, Md.

[4] *Reprint requests to:* William R. Sloan, MD, The James Buchanan Brady Urological Institute, The Johns Hopkins Hospital, Baltimore, Md 21205.

an attempt to partially circumvent this impass, we have developed a new animal model for studying the effects of nonhormonal drugs on the androgen-induced growth of the rat prostate.

An increase in prostatic growth reflects two general components, hypertrophy and hyperplasia. Hypertrophy indicates an increase in cell size and requires RNA and protein synthesis. Hyperplasia is represented by an increase in total cell number and requires DNA synthesis and a net accumulation in DNA. Relative to many other human tumors, prostatic cancer grows at a rather slow rate; nevertheless, DNA synthesis is required in the hyperplastic element of this malignancy. This paper describes a new model for assaying the effects of several common cancer chemotherapeutic agents on the rate of DNA synthesis in the ventral prostate of castrated rats after androgen-induced growth.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats weighing 250–300 g were obtained from Huntingdon Farms (West Conshohocken, Penn) and were maintained on a diet of Purina laboratory chow and water ad libitum. Animals were housed in individual metabolic cages in a temperature-controlled room under a constant 12-hour light/12-hour dark lighting schedule. Orchiectomy was performed under ether anesthesia via the scrotal route. The animals were weighed and randomly distributed into groups consisting of six to ten animals for each dose of the drugs tested.

Drug Injections

Testosterone propionate was dissolved in sesame oil at a concentration of 0.3 or 2.0 mg/0.2 ml and administered subcutaneously (sc) in animals 7 days after castration. 5-Fluorouracil (5-FU), vincristine, and cyclophosphamide were standard injectable preparations administered intraperitoneally (ip). 5-(3,3-Dimethyl-1-triazeno)imidazole-4-carboxamide (NSC-45388), CCNU, hydroxyurea, streptozotocin, adriamycin, estramustine phosphate, estradiol, and cyproterone acetate were also administered ip.

Rate of DNA Synthesis

The rate of DNA synthesis in the rat ventral prostate was determined by treating animals 7 days after castration with 2.0 mg/day of testosterone propionate for 3 days. The ventral prostate was removed from the animals 72 hours after the first injection and 24 hours after the final injection (Day 3). The tissue was minced with surgical scissors and approximately 50 mg wet weight of tissue was added to 10 ml of an incubation system of Krebs-Ringer phosphate buffer containing 122 mM NaCl, 3 mM KCl, 1 mM $MgSO_4$, 10 mM glucose, 17.5 mM NaH_2PO_4, and 10 μM thymidine (methyl-^{3}H)-thymidine, 40 microcuries. When 5-FU was used as a drug, the label was ^{14}C-sodium formate. The tissue was incubated at 37°C for 60 minutes with gentle shaking and with oxygen as a gas phase. The reaction was terminated with the addition of 5 ml of chilled incubation medium containing 0.1% (wt/vol) unlabeled thymidine or sodium formate. Tissue suspension was then centrifuged at 10,000 × g for 5 minutes at 0°C. The pellet was washed twice with 5 ml of 0.22 M perchloric acid and twice with 3 ml of ether-ethanol (1:3) containing 1% potassium acetate. The RNA was then removed from this washed pellet by base hydrolysis with 2 ml of 0.3 M KOH at 37°C for 90 minutes. After reprecipitation with 3 ml of 1.6 M perchloric acid, the DNA in the pellet was hydrolyzed by adding 2.0 ml of 1.6 M perchloric acid and heating for 20 minutes at 70°C. The hydrolyzed DNA suspension was centrifuged at 13,000 × g for 10 minutes and 0.1 ml of the supernatant was removed for DNA analysis and 0.5 ml for determination of radioactivity by addition to 14.5 ml of Hydromix from Yorktown Research Company. The counting efficiency was approximately 35% for ^{3}H and approximately 98% for ^{14}C when determined in a Beckman LS230 Scintillation counter.

It was also possible to make the above determinations in Difco-199 incubation medium in place of the Krebs-Ringer buffer. With the Difco medium, a 95% oxygen and 5% CO_2 gas phase was used. The description of these

methods and their correlation with in vivo DNA synthesis and net accumulation of DNA were first described by Coffey et al (5) and later modified by Coffey and Sufrin (6).

Total DNA Content in Prostatic Tissue

The total content of DNA in the prostate was determined by the method of Hutchinson et al (7) as applied to the rat ventral prostate by Coffey et al (5). The DNA assays in the acid hydrolysis were determined by the Burton diphenylamine method with calf thymus DNA as the reference material (8).

RESULTS

The increase in total DNA content is the most direct index of an increase in the number of cells in the prostate gland (5). Moreover, the total DNA content of the ventral prostate gland of the rat has been correlated with the size and wet weight of the rat ventral prostate (5). Figure 1 (calculated and graphed from data presented by Coffey et al [5]) shows that castration of the adult male rat causes a marked decrease in the total amount of DNA in the prostate. However, administration of exogenous testosterone propionate for 1 week is capable of restoring the total prostatic DNA content to normal levels which have been observed in intact animals (as presented in the top part of fig 1). This restoration of total DNA content is accompanied by a large increase in the rate of DNA synthesis which is maximal 3 days after the initial injection of testosterone propionate (as presented in the lower part of fig 1). The castrated animals are given daily doses of testosterone propionate in the periods (arrows) indicated in figure 1; the ventral prostate is removed and the rate of DNA synthesis is determined by incubation of the minced tissue with ^{3}H-thymidine. The increase in the specific activity of the isolated prostatic DNA is an index of the rate of DNA synthesis and is reported as counts per minute (cpm) of ^{3}H-thymidine incorporated per 100 μg of isolated DNA per hour of incubation. The profile of the rate of DNA synthesis determined by this in vitro assay system and that obtained by administering ^{3}H-thymidine in vivo are essentially identical. It is important to note that prostatic tissues from nontreated castrated animals and from normal intact animals exhibit no significant DNA synthesis, which is a reflection of the low growth rates and cell divisions of these tissues. In addition, Sufrin and Coffey (6) have determined that daily injections of 0.2–10 mg/day of testosterone propionate administered to castrated animals give essentially the same amount of DNA synthesis in the sex accessory tissues, indicating that saturation of the rate of DNA synthesis occurs above 0.2 mg/day of testosterone propionate. Moreover, recent work in our laboratory using radioautography with these tissues has indicated that over 90% of this incorporation can be accounted for by the labeling of epithelial cells of the prostate, and the mitotic index at the time of maximal DNA synthesis (3 days after initial injection) is approximately 15%–20% (9). Maximal incorporation in vitro resulted in approximately 2000 cpm/100 μg of DNA/hour as shown in figure 1; however, with improved counting efficiencies as reported here, we are now obtaining 10,000 cpm/100 μg of DNA/hour. With this system, the castrated control rat not receiving androgen gives a value of approximately 500 cpm which indicates that the androgen-stimulated gland is increased twentyfold over the gland of the castrated control. Sufrin and Coffey (6), using minimal saturation levels of testosterone propionate (0.3 mg/day), have used this system to determine the potency of antiandrogenic drugs on the effects of DNA synthesis in the rat ventral prostate. When the dose of exogenous androgen was raised to 2.0 mg/day, the effects of the antiandrogens on DNA synthesis could be negated because of their inability to compete with the high dose of androgen. We report here the use of this model in determining the effects of several well-known cancer chemotherapeutic agents on inhibition of androgen-induced DNA synthesis in the rat ventral prostate. For these studies we have utilized the higher dose of testosterone propionate of 2.0 mg/day given for 3 days simultaneously with the indicated drugs. The animals were killed 3 days after hormone and drug therapy to determine the rate of DNA synthesis in vivo. Table 1 reports the effects of several types of cancer chemotherapeutic agents. Note that the rate of DNA synthesis in

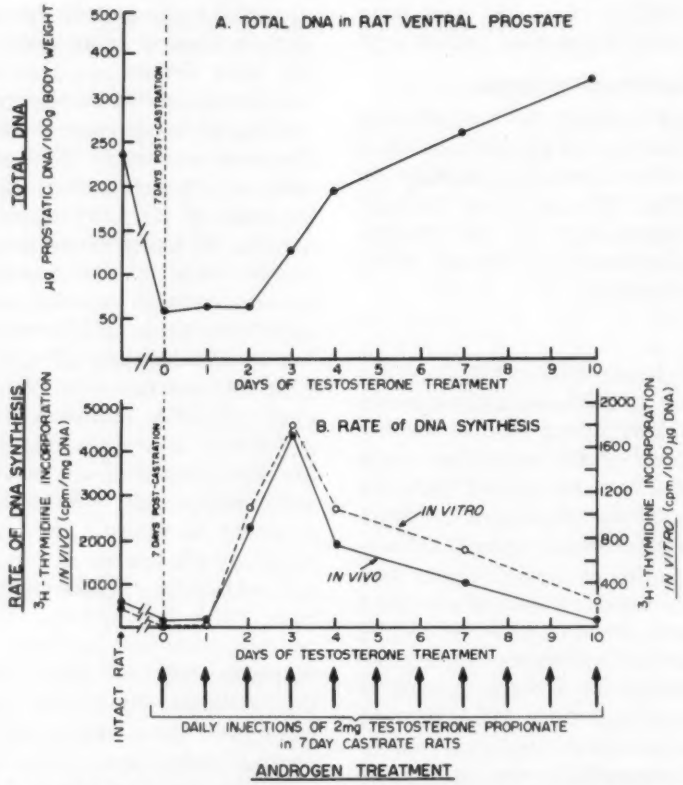
A. TOTAL DNA in RAT VENTRAL PROSTATE
TOTAL DNA
μg PROSTATIC DNA/100g BODY WEIGHT
7 DAYS POST-CASTRATION
DAYS OF TESTOSTERONE TREATMENT
B. RATE of DNA SYNTHESIS
RATE OF DNA SYNTHESIS
^{3}H - THYMIDINE INCORPORATION IN VIVO (cpm/mg DNA)
^{3}H - THYMIDINE INCORPORATION IN VITRO (cpm/100 μg DNA)
IN VITRO
IN VIVO
INTACT RAT
DAILY INJECTIONS OF 2mg TESTOSTERONE PROPIONATE in 7 DAY CASTRATE RATS
ANDROGEN TREATMENT

FIGURE 1.—Effects of castration and exogenous androgen administration on: (A) total DNA content in the rat ventral prostate and (B) rate of DNA synthesis determined by ^{3}H-thymidine incorporation when administered in vivo and by incubation of ^{3}H-thymidine for 1 hr with minced ventral prostatic tissue (in vitro). DNA was isolated from the tissue and the specific activity of the ^{3}H-thymidine incorporation was determined as described in the Methods section. Graph from data presented by Coffey et al (5).

the castrated controls is stimulated approximately 25-fold by the administration of either 0.3 or 2.0 mg of testosterone propionate. The data are compared relative to the higher dose of testosterone propionate (2.0 mg/day for 3 days) and are set at 100 which is equivalent to approximately 11,000 cpm/100 μg of DNA/hour. The androgen was given sc and the drugs were given ip. Cyclophosphamide has no effect on the rate of DNA synthesis even when administered at extremely high doses of 80 mg/kg/day (a highly toxic level). Similar observations were made with CCNU; however, estramustine phosphate (a derivative of estradiol) with an alkylating group did inhibit DNA synthesis significantly but this was observed only at doses of 0.3 mg/day of testosterone propionate and the variation was large. The antimetabolite, 5-FU, was very effective at high doses, and this could be determined by using ^{14}C-sodium formate as the precursor for DNA synthesis or by using ^{3}H-thymidine. One would anticipate that it would be important to use the formate label since 5-FU inhibits thymidylate synthetase which is in the de novo pathway, and the thymidine incorporation is via a salvage

pathway circumventing this reaction. However, significant inhibition was observed with both labels. The other effective drugs were adriamycin and hydroxyurea. Streptozotocin was without effect when given simultaneously with androgens, but Sufrin and Prutkin (10) have reported that if animals are made diabetic with this agent prior to the administration of testosterone propionate, the androgen-induced DNA synthesis is inhibited approximately 50%. This effect can be reversed, however, by the administration of insulin. The effects of cyproterone acetate are observed only at the lower doses of testosterone propionate where the antiandrogen competes with the limiting level of the androgen. Estradiol is not capable of blocking the DNA synthesis when given in the presence of either low or high doses of the androgen, and these effects of the hormone have been fully described in the study by Sufrin and Coffey (6). Vincristine is a mitotic inhibitor which might be anticipated to block prostatic growth subsequent to the S phase and thus would not be detected as an inhibitor of DNA synthesis. To determine this we administered a high dose of vincristine for 7 days to measure its effect on the wet weight of the rat ventral prostate. It was indeed diminished significantly but not remarkably (*see* fig 2).

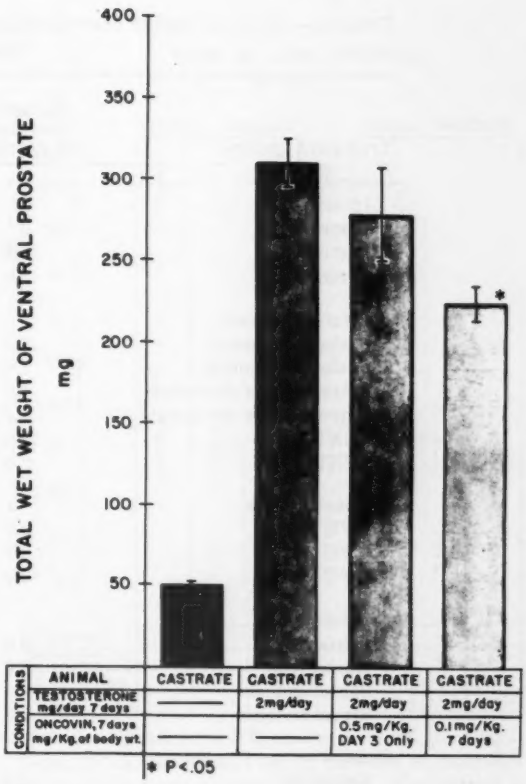

FIGURE 2.—Effects of vincristine on the androgen-induced growth of the rat ventral prostate as determined by increases in wet weight of the gland. Bars represent standard errors of the mean.

To evaluate this model as a means of determining the effects of cancer chemotherapeutic drugs on growth and DNA synthesis, we studied one of the more effective agents, 5-FU, and compared it to the effects observed with cyclophosphamide which was not highly effective in blocking DNA synthesis. Figure 3 represents a dose-response curve observed with 5-FU and its ability to inhibit DNA synthesis in the rat ventral prostate determined with ^{14}C-sodium formate incorporation into DNA.

There are differences in the embryonic development of the various lobes of the rat prostate and there has been much speculation as to which lobe might correlate with that of the outer portion of the human prostate gland which is the site of prostatic adenocarcinoma development (11). It was therefore important to determine if 5-FU is capable of inhibiting DNA synthesis in the dorsal lobe of the prostate, and this inhibition is compared to that of the ventral lobe in figure 4.

The inhibition observed with 5-FU on the rate of DNA synthesis must correlate with its ability to block the overall increase and growth of the gland as determined by its increase in gross wet weight. The effect of 5-FU on the wet weight of the ventral and dorsal prostate is presented in figure 5, and it is apparent that it is capable of blocking such growth.

Cantarow and Zagerman (12) have reported that 5-FU might have antiandrogenic properties on the seminal vesicles of the rat. The absence of an antiandrogenic effect in our system was established by comparing 5-FU with the well-known antiandrogen, cyproterone acetate, given with both low and high doses of testosterone propionate (fig 6). It is apparent that the

TABLE 1.—Effects of cancer chemotherapeutic agents on DNA synthesis in rat ventral prostate*

Treatment groups	Testosterone propionate (mg/day × 3)	Drug dose (mg/kg/ day × 3)	Ventral prostate relative DNA synthesis (% on Day 3 ± SE)†
Controls			
Intact	0	0	7 (±4)
Castrated	0	0	4 (±2)
Castrated	0.3	0	93 (±11)
Castrated	2.0	0	100 (±15)
Alkylating agents			
Cyclophosphamide	2.0	20	93 (±11)
Cyclophosphamide	2.0	80	105 (±9)
Estramustine phosphate	0.3	10	73 (±14)
Estramustine phosphate	0.3	40	50 (±33)
CCNU	2.0	10	88 (±12)
CCNU	2.0	40	85 (±18)
Antimetabolites			
5-FU	2.0	7.5	96 (±8)
5-FU	2.0	30	70 (±12)
5-FU	2.0	60	40 (±10)
Antibiotics			
Adriamycin	2.0	2	50 (±13)
Adriamycin	2.0	11	33 (±13)
Streptozotocin	2.0	25	104 (±8)
Streptozotocin	2.0	75	100 (±14)
Hormones			
Estradiol	0.3	10	86 (±5)
Estradiol	2.0	10	80 (±7)
Cyproterone acetate	0.3	5	21 (±6)
Cyproterone acetate	2.0	5	89 (±9)
Miscellaneous			
Hydroxyurea	2.0	100	28 (±10)
NSC-45388	2.0	200	102 (±7)
Vincristine	2.0	0.3	100 (±8)

*Effects of various drugs in inhibiting the rate of DNA synthesis in the rat ventral prostate from castrated rats receiving testosterone propionate for 3 days. Seven days after castration each animal received the indicated doses of testosterone propionate and the drug for 3 days. The ventral prostate was minced and incubated with ^{3}H-thymidine or ^{14}C-sodium formate (in the case of 5-FU). The specific activity of the isolated DNA was determined.

†100% = 1.1×10^{4} cpm/100μg of DNA/hr.

higher dose of testosterone propionate is capable of reversing the antiandrogenic effects of cyproterone acetate but is essentially without effect on the inhibition observed with 5-FU.

If 5-FU brings about its effects in inhibiting prostatic growth by blocking DNA synthesis, one might anticipate that the treatment of the normal rat with 5-FU would not diminish the size of the prostate gland because there is very little DNA synthesis in the prostate of the normal intact rat. This is presented in figure 7 (Part A) and it is apparent that 5-FU does not decrease the size of the ventral prostate, dorsal prostate, or seminal vesicles in the intact animal. In contrast it is shown in figure 7 (Part B) that 5-FU is capable of blocking androgen-stimulated growth in castrated animals in all of these glands.

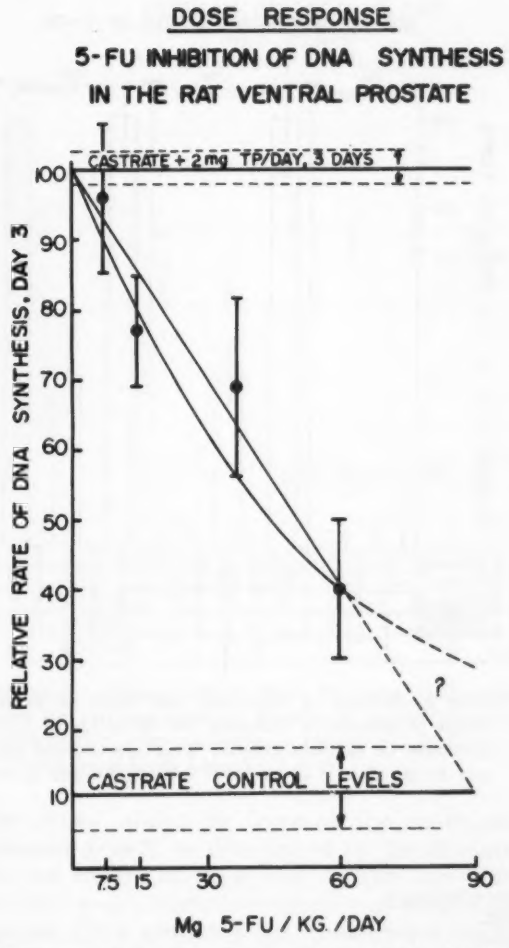

FIGURE 3.—Dose-response curve of the effects of 5-FU on inhibiting the androgen-induced rate of DNA synthesis determined in vitro. Seven days after castration, rats were treated with 2 mg/day of testosterone propionate for 3 days and received simultaneous injections of varying doses of 5-FU. On Day 3, 72 hrs after the initial injection and 24 hrs after the final injection, the ventral prostate was removed and incubated with ^{14}C-sodium formate and the specific activity of the isolated DNA was determined. Bars represent standard errors of the mean.

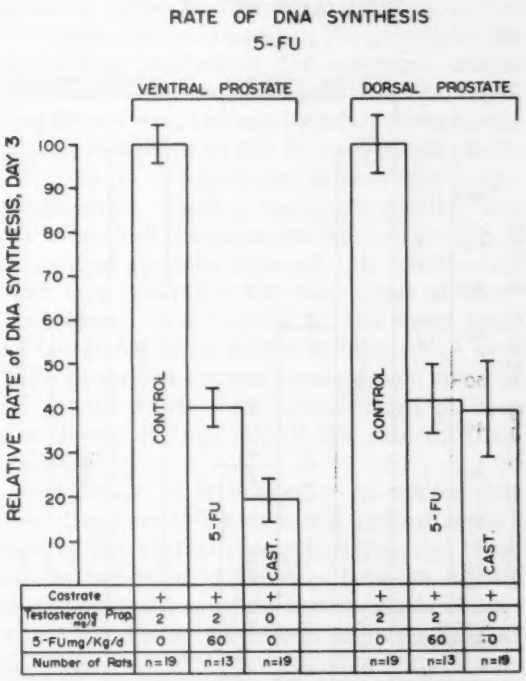

FIGURE 4.—Effects of 5-FU on the rate of DNA synthesis in the ventral and dorsal prostate of castrated rats receiving testosterone propionate. The method is that described in fig 3 and in the Methods section.

The alkylating agent, cyclophosphamide, was not effective in blocking the androgen-induced DNA synthesis in the castrated rat ventral prostate. It was also established that even at very toxic doses this drug was not capable of blocking the androgen-induced increase in the wet weight of the rat ventral prostate (fig 8).

DISCUSSION

We have a new model for determining the effects of nonhormonal agents on the androgen-induced growth of the ventral prostate in castrated rats. This model utilizes the ability of cancer chemotherapeutic agents to block the androgen-stimulated rate of DNA synthesis observed in this tissue. It is apparent that there is a high degree of selectivity between the various types of therapeutic agents and their ability to inhibit DNA synthesis in this tissue. The most effective agents are 5-FU, adriamycin, and hydroxyurea. In contrast, many other agents including cyclophosphamide, CCNU, streptozotocin, vincristine, and NSC-45388 show no significant effects. The ability to block the rate of DNA synthesis could be correlated with

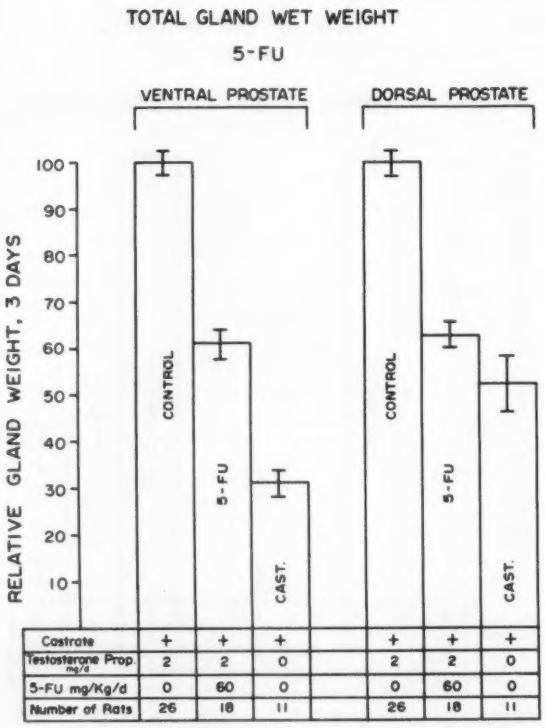

FIGURE 5.—Effects of 5-FU on the androgen-induced increase in wet weight of the ventral and dorsal prostate of castrated rats. The method is that described in fig 3 with the exception that the gland weight was determined.

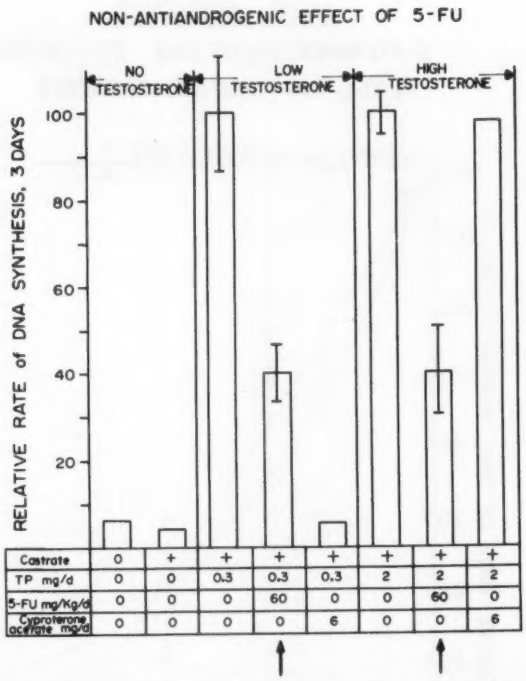

FIGURE 6.—Effects of high and low doses of testosterone propionate in reversing the inhibition of DNA synthesis in animals treated with cyproterone acetate (6 mg/day × 3) or 5-FU (60 mg/kg/day × 3).

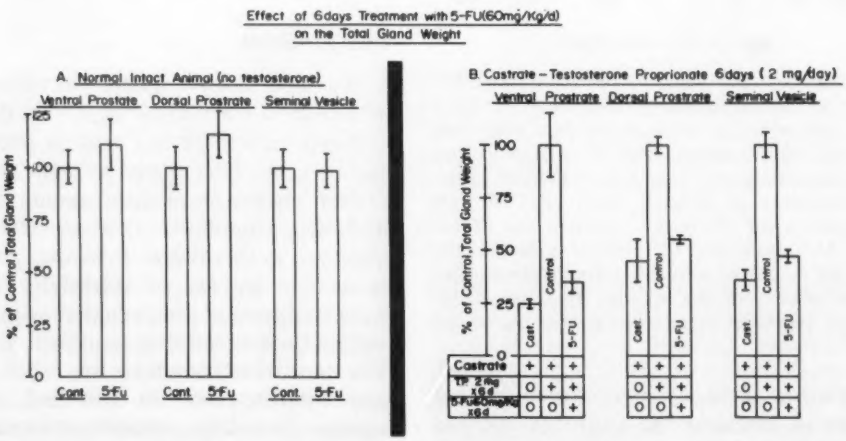

FIGURE 7.—Comparison of the effects of 5-FU on the size of the ventral prostate, dorsal prostate, and seminal vesicles in normal intact animals (*A*) and in castrated animals (*B*) receiving exogenous testosterone propionate. All animals were treated for 6 days.

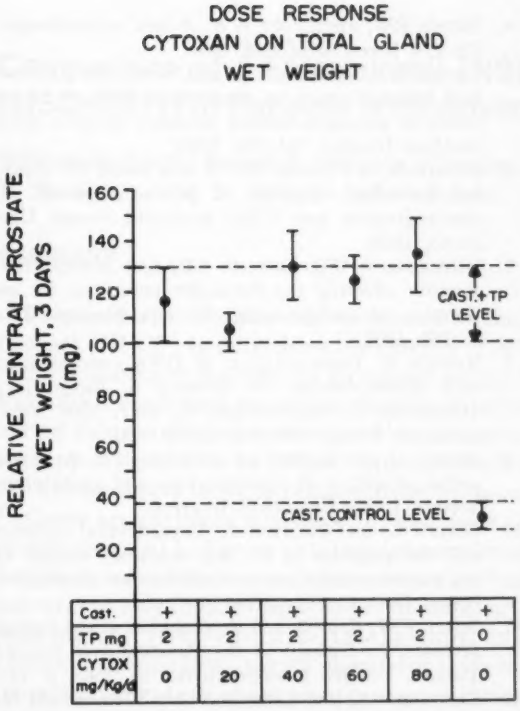

FIGURE 8.—Absence of an effect of cyclophosphamide on the androgen-induced growth of ventral prostate observed in castrated animals receiving 2 mg/day of testosterone propionate for 3 days.

the drugs' ability to decrease the androgen-induced growth as determined by the changes in wet weight of these organs. The exception to this was vincristine which was unable to inhibit DNA synthesis but caused some slight decrease in the wet weight of the organ. This latter effect might be anticipated because of the mode of action of vincristine as a mitotic inhibitor which is subsequent to the increase in DNA synthesis.

It is well known that many cancer chemotherapeutic agents have different degrees of efficacy on different types of tumor systems. We believe that it is important to ascertain the selectivity of these drugs in blocking the growth of prostatic tissue. It is hoped that some of the information might be of importance in determining how these drugs might affect prostatic growth in other species. It must be recognized that there are considerable species differences in the effects of any drug, and it might be difficult to extrapolate to what might be observed in man. Furthermore, prostatic cancer is a slow-growing tumor and the growth of the rat ventral prostate in the castrated animal receiving exogenous androgens is quite rapid. This system would primarily select those agents causing inhibition in the G_1 or S phase of the cell cycle. It is important to note that cyclophosphamide, which is not cycle specific, was unable to block the androgen-induced growth in the ventral prostate, although this could not be taken as a generality for other types of alkylating drugs. The toxicity of the drug could not be related to its ability to block DNA synthesis in the rat ventral prostate, and some of the agents which were administered at very toxic levels did not inhibit the rate of DNA synthesis.

In addition, it is important to realize that these drugs are being measured against rapidly growing rat ventral prostatic tissue, and there may be important differences between rapidly growing normal prostate and prostatic cancer. It is nevertheless visualized that this type of information might provide some insight into regulating abnormal prostatic growth. At present, there is no acceptable model for prostatic cancer in animals. When such a system is developed, it would be very informative to compare this type of data with the effects of these drugs on abnormal prostatic tissue.

LIST OF COMPOUNDS

Adriamycin: NSC-123127. Supplied by Cancer Therapy Evaluation (CTE), Division of Cancer Treatment (DCT), National Cancer Institute (NCI).

CCNU: NSC-79037; CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-. Supplied by CTE, DCT, NCI.

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan. Supplied by Mead Johnson Laboratories.

Cyproterone acetate: NSC-81430; CAS reg. No. 427-51-0; pregna-4,6-diene-3,20-dione, 6-chloro-17-hydroxy-1α, 2α-methylene-, acetate. Supplied as a gift from Schering AG Berlin.

Estradiol: NSC-9895; CAS reg. No. 50-28-2; estra-1,3,5-(10)-triene-3,17β-diol; 17-β-estradiol. Supplied by Sigma Chemical Co.

Estramustine phosphate: NSC-89199; CAS reg. No. 4891-15-0; estra-1,3,5(10)-triene-3,17β-diol, 3-[bis(2-chloroethyl)carbamate] 17-(dihydrogen phosphate);

Estracyt. Supplied by A. E. Leo Co. through the courtesy of Gerald P. Murphy, MD, Roswell Park Memorial Institute, Buffalo, NY.

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8; 5-FU. Supplied by Roche Laboratories.

Hydroxyurea: NSC-32065; CAS reg. No. 127-01-07. Supplied by CTE, DCT, NCI.

NSC-45388: CAS reg. No. 4342-03-4; DTIC. Supplied by CTE, DCT, NCI.

Streptozotocin: NSC-85998. Supplied by CTE, DCT, NCI.

Testosterone propionate: NSC-9166. Supplied by Sigma Chemical Co.

Vincristine: NSC-67574; CAS reg. No. 6449-03-2; leurocristine, sulfate (1:1); Oncovin. Supplied by Eli Lilly and Co.

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Comparison of 5-Fluorouracil (NSC-19893) and Cyclophosphamide (NSC-26271) in Patients With Advanced Carcinoma of the Prostate [1,2]

William W. Scott,[3] Robert P. Gibbons,[4] Douglas E. Johnson,[5] George R. Prout,[6] Joseph D. Schmidt,[7] T. M. Chu,[8] John F. Gaeta,[8] James Joiner,[8] Jack Saroff,[8] and Gerald P. Murphy [8,9,10]

SUMMARY

This cooperative study was sponsored by the National Prostatic Cancer Project (NPCP) to determine the efficacy of chemotherapy compared to current standard therapy for advanced (stage D) prostatic carcinoma. Patients are selected for randomization to an initial 12-week treatment period using either cyclophosphamide, 5-fluorouracil (5-FU), or standard therapy. All patients have histologically confirmed prostatic cancer and are unresponsive to, or in relapse after, hormonal or ablative therapy. Patients are excluded who have (*a*) had more than 2000 rads of pelvic irradiation, (*b*) had prior cytotoxic chemotherapy, (*c*) a second primary neoplasm except for nonmelanomatous skin tumors, (*d*) an expected survival of less than 90 days, or (*e*) hematologic evidence of an insufficient bone marrow reserve for cytotoxic therapy. Patients are monitored by clinical examinations, serum chemistry tests including acid and alkaline phosphatase values, chest roentgenograms, intravenous pyelograms, skeletal surveys, and determinations of subjective response. All available histologic material is reviewed by referee pathologists at Roswell Park Memorial Institute. Phosphatase values are corroborated by analyses of serum samples submitted to a central laboratory at Roswell Park Memorial Institute. All data and results are tabulated and coordinated by the Central Statistical Office of the NPCP. At the end of the initial 12-week period the patient's tumor status is re-evaluated objectively for complete or partial regression, stability, or progression. Only patients receiving 5-FU or cyclophosphamide whose disease status progresses are given the alternate cytotoxic agent. All patients are followed until death. From July 1, 1973, to May 15, 1974, 66 patients were entered in the study. Of these, 34 patients have been in the study for 12 weeks or longer. From

[1] Supported in part by Public Health Service grants CA-15407, CA-15421, CA-15107, CA-15284, CA-15108, and CA-15126 from the National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[2] 5-Fluorouracil: CAS reg. No. 51-21-8; 5-FU.

Cyclophosphamide: CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan.

Both drugs were supplied by Cancer Therapy Evaluation, Division of Cancer Treatment, NCI.

[3] Johns Hopkins Hospital, Baltimore, Md.

[4] Virginia Mason Medical Center, Seattle, Wash.

[5] M. D. Anderson Hospital and Tumor Institute, University of Texas, Houston.

[6] Massachusetts General Hospital, Boston.

[7] University of Iowa Hospitals and Clinics, Iowa City.

[8] Roswell Park Memorial Institute, Buffalo, NY.

[9] Co-investigators include *William F. Catalona* (Johns Hopkins Hospital); *Roy J. Correa* and *Kenneth B. Cummings* (Virginia Mason Medical Center); *Roman Chalbaud* (M. D. Anderson Hospital and Tumor Institute); *Hark C. Chang* (Massachusetts General Hospital); and *Christine Webster* (University of Iowa Hospitals and Clinics).

[10] *Reprint requests to*: William W. Scott, MD, Department of Urology, Johns Hopkins Hospital, Baltimore, Md 21205.

this group of patients, nine were originally assigned to receive 5-FU, 14 to receive cyclophosphamide, and 11 to receive standard therapy. Preliminary results suggest a more favorable response for cyclophosphamide compared to 5-FU or standard therapy, ie, 21% partial tumor regression versus 11% and 0 respectively. Subjective pain relief occurred in 56% of the 5-FU-treated patients, 57% of the cyclophosphamide-treated patients, but only 27% of the patients receiving standard therapy. Severe hematologic toxicity was observed in two patients receiving 5-FU and one patient receiving cyclophosphamide. Severe gastrointestinal toxicity was observed in three patients (21%) only, all of whom received cyclophosphamide. These results plus results for duration of remission, dose modification, and crossover therapy will be discussed in this paper. It should be emphasized that these are very preliminary results. Nevertheless, they are encouraging in that the incidence of favorable responses and the acceptable degree of toxicity observed using these drugs indicate that one or both may be considered legitimate therapeutic options for patients with endocrine-resistant prostatic carcinoma.

[Cancer Chemother Rep 59:195–201, 1975]

During the past year several controlled clinical trials have been initiated to evaluate the efficacy of nonhormonal chemotherapy in the treatment of prostatic carcinoma. The National Prostatic Cancer Project (NPCP) has sponsored one such trial, a cooperative phase II study of cyclophosphamide and 5-fluorouracil (5-FU) in patients who have relapsed after standard endocrine therapy. These two drugs were chosen for study because previous laboratory (1) and clinical (2)[11] investigations suggested their potential effectiveness. It should be understood that the report that follows represents a study still in progress and is therefore preliminary in nature.

MATERIALS AND METHODS

Patient Selection

Patients selected for this study have histologically confirmed prostatic carcinoma and are unresponsive to or in relapse after hormonal or ablative therapy. Patients are excluded who have (*a*) had more than 2000 rads of radiation therapy to the pelvis, (*b*) had prior cytotoxic chemotherapy, (*c*) a second primary neoplasm except for nonmelanomatous skin tumors, (*d*) an expected survival of less than 90 days, or (*e*) hematologic evidence of an insufficient bone marrow reserve for cytotoxic therapy (white blood cell count < 4000 cells/mm^3 and platelet count $< 100{,}000$ cells/mm^3).

Randomization

Patients are randomly assigned by the NPCP Central Statistical Office (based at Roswell Park Memorial Institute [RPMI]) to receive either 5-FU, cyclophosphamide, or standard therapy (fig 1). The latter treatment may include any nonchemotherapeutic treatment. Hormonal therapy is discontinued in patients randomized to 5-FU or cyclophosphamide 14 days before being placed in the study.

Onstudy Patient Care

Patients are monitored weekly, when feasible, with clinical examinations and blood studies, including serum acid phosphatase determinations. Serum samples are sent to RPMI for duplicate acid phosphatase determinations as well as other special studies. Chest roentgenograms, intravenous pyelograms, and skeletal surveys are obtained upon entry into the study and at 6-week intervals. All available histologic material is submitted to RPMI and is reviewed by referee pathologists.

[11] Slavik M, and Carter SK. Single agents in prostatic cancer—a quick review. Prepared by the NCI, Bethesda, Md, for the NPCP.

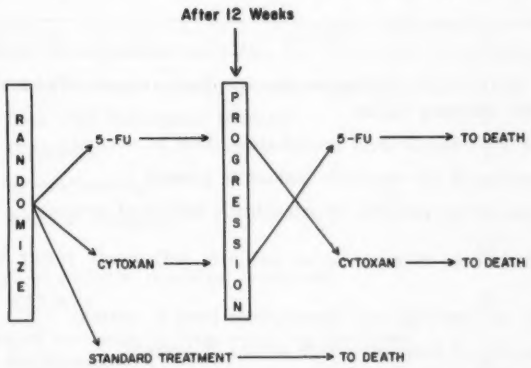

FIGURE 1.—Treatment schema: 5-FU (600 mg/m²/week intravenously); cyclophosphamide (1 g/m² intravenously every 3 weeks). Crossover treatment is given when progression occurs unless death intervenes.

Duration of Study

Patients are assigned to one treatment group for a minimum of 12 weeks. At the end of this period, the tumor status of the patient is re-evaluated and classified relative to the baseline status as either (*a*) complete tumor regression, (*b*) partial tumor regression, (*c*) stable, or (*d*) tumor progression, according to the criteria shown in table 1. Those patients manifesting objective tumor progression while receiving one of the cytotoxic drugs are crossed over to the other drug after 12 weeks, while those not manifesting tumor continue to receive their original therapy until such progression becomes evident. Patients assigned to standard therapy continue to receive such therapy for the duration of the study. All patients are followed until death.

RESULTS

Original Therapy

As of May 15, 1974, 66 patients were entered into the study (table 2). Of these, 13 were removed from therapy for the reasons indicated and 19 have been receiving treatment less than 12 weeks; consequently the cases of these patients are not evaluated in this report. Thus, we will confine our remarks to the preliminary results observed among 34 patients receiving treatment for a minimum of 12 weeks.

Because all patients manifested active tumor progression at the time of entry into the study, those who either remained stable or had tumor regression were considered to have a favorable response (table 3). These preliminary tabulations suggest that cyclophosphamide-treated patients fared better than their counterparts receiving 5-FU or standard therapy.

Of this group, nine were originally assigned to 5-FU, 14 to cyclophosphamide, and 11 to standard therapy. Among those assigned to 5-FU, one (11%) had partial tumor regression, two (22%) remained stable, and six (67%) had tumor progression. In contrast, three (21%) of the cyclophosphamide-treated patients had partial regression, six (43%) remained stable, and five (36%) had tumor progression. No patient receiving standard therapy had partial tumor regression, four (33%) remained stable, and seven (59%) had tumor progression. The higher incidence of favorable responses with cyclophosphamide therapy compared to both 5-FU and standard therapy should be noted. No apparent difference was observed between the results of 5-FU and standard therapy.

The duration of remissions (partial regression and stable) occurring in the various treatment groups is shown in table 4. Of three patients with remissions from 5-FU, one manifested tumor progression after 19 weeks of treatment while two continue in remission after 40 and 43 weeks respectively. Of nine patients with remission from cyclophosphamide, two had tumor progression after 23 and 27 weeks of treatment. One patient in tumor remission was withdrawn from cyclophosphamide therapy after 13 weeks of severe toxicity, while six others remain in remission after 25–44 weeks of such therapy. Two of four patients receiving standard therapy had tumor progression after 17 and 24 weeks of treatment, while two receiving standard therapy have remained in remission for 18 and 32 weeks.

Crossover Therapy

The early results of crossover therapy in 5-FU- and cyclophosphamide-treated patients manifesting tumor progression from their initial treatment are shown in table 5. When therapy was crossed over from 5-FU to cyclo-

TABLE 1.—NPCP chemotherapy protocol: evaluation of therapy

Response	Criteria
Complete objective regression	*a.* Absence of any clinically detectable soft tissue tumor; this may include the primary tumor
	b. Return of an elevated acid phosphatase level to normal
	c. Recalcification of all osteolytic lesions if present
	d. No evidence of progression of osteoblastic lesions if present
Partial objective regression	*a.* 50% reduction in measurable or palpable soft tissue tumor if present
	b. Return of an elevated acid phosphatase level to normal
	c. Recalcification of some osteolytic lesions if present
Complete and partial objective regression	*a.* No increase in any other lesion and no new areas of malignant disease
	b. No significant deterioration in weight (>10%), symptoms, or performance status (1 level)
	c. If hepatomegaly is a significant indicator, there must be a reduction in liver size and at *least* a 30% improvement of all pretreatment abnormalities of liver function
Objectively stable	All of the following:
	a. Insufficient regression of primary indicator lesion to meet above criteria
	b. <25% increase in any measurable lesion
	c. No significant deterioration in weight (>10%), symptoms, or performance status (1 level)
Objective progression (or relapse after adequate therapy)	Any of the following:
	a. Significant deterioration in symptoms, decrease in weight, or decrease in performance status
	b. Appearance of new areas of malignant disease
	c. Increase in any previously measurable lesion (soft tissue and lung, exclude bone) by >50% in two perpendicular diameters
	d. Increase in acid or alkaline phosphatase level *alone* is not considered an indication of progression; this should be used in conjunction with other criteria

phosphamide, one patient showed an arrest of tumor progression while six manifested continued tumor progression. Similarly, in one patient crossed over from cyclophosphamide to 5-FU tumor progression was arrested while three had continued progression. These very preliminary observations would seem to suggest that sequential therapy with these agents is of limited additional benefit for patients with resistant tumors.

Subjective Responses

Patients were also evaluated for favorable subjective responses to therapy (table 6). All patients included in these tabulations had pain upon entry into the study. Subjective pain relief occurred in 56% of the 5-FU-treated patients, 57% of the cyclophosphamide-treated patients, but only 27% of the patients receiving standard therapy. These differences are not statistically significant.

TABLE 2.—NPCP chemotherapy protocol: patient accrual*

Total No. of patients entered	66
No. of patients removed and not evaluable	
Died with inadequate therapy	5
Withdrew	3
Severe toxicity	3
Protocol violation	2
	13
No. of patients receiving treatment <12 wks	19
No. of evaluable patients receiving treatment for 12 wks	34

*Accrual from July 1, 1973, to May 15, 1974.

Toxicity

The dose schedules of 5-FU and cyclophosphamide are sufficiently different so that a strict comparison of their relative toxicities would not be valid. Further, patients receiving 5-FU therapy tend to be seen more frequently (because they are treated weekly) than cyclophosphamide-treated patients who receive treatments once every 3 weeks. Consequently, patients receiving 5-FU therapy have an increased opportunity for the detection of bone marrow toxicity. For these reasons, no attempt at a comparative assessment of toxicity was made.

For this review, two general categories of

TABLE 3.—NPCP chemotherapy protocol: tumor response to therapy*

Treatment	No. of patients with—			Total No. of patients
	Partial regression	Stable disease	Progression	
5-FU	1 (11%)	2 (22%)	6 (67%)	9
Cyclophosphamide	3 (21%)	6 (43%)	5 (36%)	14
Standard	0 (0)	4 (36%)	7 (64%)	11

*Response determined at 12 wks; accrual from July 1, 1973, to May 15, 1974.

TABLE 4.—NPCP chemotherapy protocol: duration of remission

Treatment	Total No. of patients in remission at 12 wks*	Duration of treatment before progression occurred (wks)	Duration of remission for those not yet progressing† (wks)
5-FU	3 (33%)	19‡	40,43
Cyclophosphamide	9 (64%)	23,27	"13," 25,25,28, 32,33,44
Standard	4 (36%)	17,24	28,32

*Includes partial remission and stable response.
†Patients in remission as of May 15, 1974.
‡ Nos. underlined indicate patients with partial regression.

TABLE 5.—NPCP chemotherapy protocol: response to crossover therapy*

Treatment	No. of patients with—		Total No. of patients
	Tumor regression or stable disease	Tumor progression	
5-FU to cyclophosphamide	1 (14%)	6 (86%)	7
Cyclophosphamide to 5-FU	1 (25%)	3 (75%)	4

*Treatment crossover prior to May 15, 1974.

TABLE 6.—NPCP chemotherapy protocol: pain relief*

Treatment	No. of patients with—		Total No. of patients
	Pain relief	No pain relief	
5-FU	5 (56%)	4 (44%)	9
Cyclophosphamide	8 (57%)	6 (43%)	14
Standard	3 (27%)	8 (73%)	11

*Treatment given for 12 wks; accrual from July 1, 1973, to May 15, 1974.

toxicity from the initial therapy were evaluated: (*a*) hematologic, defined as a white blood cell count of less than 4000 cells/mm^3 or a platelet count of less than 100,000 cells/mm^3; and (*b*) gastrointestinal, including nausea, vomiting, diarrhea, bleeding, and liver function abnormalities.

As may be observed in table 7, hematologic toxicity was observed in five of nine (55%) of the patients receiving 5-FU therapy but was severe in only two (22%). Gastrointestinal toxicity was observed in seven of nine 5-FU-treated patients (78%) but was severe in none.

Hematologic toxicity was documented in four of 14 (28%) of the patients treated with cyclophosphamide but was severe in only one (7%) (table 8). Gastrointestinal toxicity was observed in 13 (93%) of the patients and was severe in three (21%).

The dose schedule was modified in eight (89%) of the patients receiving 5-FU (table 9). However, the vast majority of such modifications were transient reductions in dose due to mild leukopenia. By contrast, the dose schedule was modified in only 36% of the patients treated with cyclophosphamide. These figures belie the fact that cyclophosphamide is generally known to be associated with more severe bone marrow and gastrointestinal toxicity than is 5-FU.

DISCUSSION

It should be emphasized that these data are very preliminary and should be regarded as

TABLE 7.—NPCP chemotherapy protocol: toxicity from 5-FU*

Toxicity	Degree of toxicity (No. of patients)			Total No. of patients†
	None	Moderate	Severe	
Hematologic	4	3	2	9
Gastrointestinal	2	7	0	9

*Treatment given for 12 wks; accrual from July 1, 1973, to May 15, 1974.

†No. of evaluable patients receiving 5-FU.

TABLE 8.—NPCP chemotherapy protocol: toxicity from cyclophosphamide*

Toxicity	Degree of toxicity (No. of patients)			Total No. of patients†
	None	Moderate	Severe	
Hematologic	10	3	1	14
Gastrointestinal	1	10	3	14

*Treatment given for 12 wks; accrual from July 1, 1973, to May 15, 1974.

†No. of evaluable patients receiving cyclophosphamide.

TABLE 9.—NPCP chemotherapy protocol: dose modification due to toxicity*

Treatment	No. of patients with dose schedule modifications/ total No. treated	No. of patients without dose schedule modifications/ total No. treated
5-FU	8/9	1/9
Cyclophosphamide	5/14	9/14

*Treatment given for 12 wks; accrual from July 1, 1973, to May 15, 1974.

such; however, some inferences can be drawn. While it seems certain that neither of the drugs under investigation represents a panacea for the patient with endocrine-resistant prostatic carcinoma, each has demonstrated its effectiveness in some patients. In this regard, our early tabulations would seem to indicate that cyclophosphamide is the superior drug, producing favorable responses in more than half of the patients treated.

Finally, we are encouraged by these early results for the immediate reason that the incidence of favorable responses and the acceptable degree of toxicity we have observed using these drugs indicate that one or both may be considered legitimate therapeutic options for patients with endocrine-resistant prostatic carcinoma. But, more importantly, we are encouraged that, in the absence of both a theoretical understanding of the molecular biology of prostatic carcinoma and an acceptable animal model for the study of this disease, empirical clinical studies such as this one provide a rational avenue of investigation in the search for better drugs to control prostatic cancer and, thus, to reduce the considerable morbidity and mortality wrought by this disease.

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Comparison of Adriamycin (NSC-123127) and the Combination of 5-Fluorouracil (NSC-19893) and Cyclophosphamide (NSC-26271) in Advanced Prostatic Cancer: A Preliminary Report[1]

R. T. Eagan, D. C. Utz, R. P. Myers, and W. L. Furlow[2,3]

SUMMARY

A preliminary report on chemotherapy for advanced prostate malignancy is presented. Nine patients have been entered in the study, of which eight are evaluable for tumor response. Of four patients initially treated with adriamycin, only one brief (5-week) objective response was noted in a patient with soft tissue disease. No responses have been seen in four patients treated with the combination of 5-fluorouracil and cyclophosphamide. No responses have been noted in five patients who failed initial treatment and were crossed over to the other treatment. Drug toxicity has been quite acceptable.

[Cancer Chemother Rep 59:203–207, 1975]

Cancer of the prostate is a major health problem in the United States and represents the third most common site of cancer causing death in men (after lung cancer and cancer of the colon and rectum). The estimated incidence of prostatic cancer in 1973 is 38,000 cases and the estimated number of deaths from this disease is 17,800 (1). Despite the size of the problem, little, if anything, is known about chemotherapy for this tumor.

Estrogen therapy, orchiectomy, and radiation therapy have been, and still are, the mainstays of treatment for advanced prostatic cancer. When patients fail to respond to these forms of therapy, there are no other known conventional therapies of proven value that can be used. Essentially, all conventional and investigational chemotherapeutic agents have been either inadequately tested or not tested at all.[4] From what limited data exist, it appears that cyclophosphamide, 5-fluorouracil (5-FU), and adriamycin may have some degree of activity.[4]

Because blastic skeletal disease is the only or major type of metastatic spread, the objective evaluation of response to treatment is difficult. No well-defined and thoroughly tested system has been established. The meaning of the changes which occur in the venous serum acid phosphatase level after therapy is unclear, although such changes usually reflect a response to therapy. Some recent studies (2,3) suggest that the bone marrow serum acid phosphatase level might be a more sensitive diagnostic tool than the venous serum acid phosphatase level, but its value as a parameter to be observed during therapy has not yet been studied.

This study was designed with two major purposes in mind: (*a*) to attempt to establish a system that could be used in ascertaining

[1] 5-Fluorouracil: CAS reg. No. 51-21-8; 5-FU.

Cyclophosphamide: CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan.

All drugs used in this study were supplied by Cancer Therapy Evaluation, Division of Cancer Treatment, National Cancer Institute (NCI).

[2] Mayo Clinic, Rochester, Minn.

[3] *Reprint requests to:* Robert T. Eagan, MD, Medical Oncology, Mayo Clinic, Rochester, Minn 55901.

[4] Carter SK. The integration of chemotherapy into a combined modality treatment strategy for solid tumors. Prepared by the NCI, Bethesda, Md.

Slavik M, and Carter SK. Single agents in prostatic cancer: a quick review. Prepared by the NCI, Bethesda, Md.

patient response to therapy, and (*b*) to compare two different chemotherapeutic programs incorporating the new system of data analysis. To date, this study is in its early stages and only preliminary data are presented.

MATERIALS AND METHODS

Nine men with grade II and III adenocarcinoma of the prostate have been entered in the study. All nine patients had failed both oral and intravenous (iv) estrogen therapy and eight of the nine had failed orchiectomy (one patient refused orchiectomy). None had had prior chemotherapy.

The patients' ages ranged from 54 to 69 years with a median age of 64 years. Seven of the nine men have had skeletal metastases as their major site of disease and two of these nine have had soft tissue metastases (excluding prostatic bed recurrence) as the predominant site of metastases, although both men have also had skeletal metastases. All nine patients have had some amount of prior radiation therapy. The amount of bone marrow radiated was estimated from the work of Ellis (4) as modified by us (table 1).

Figure 1 shows the eligibility criteria for entry in this study. Prior to randomization between the two drug programs, patients were stratified by three criteria:

1. age: (*a*) less than 65 years of age, (*b*) 65 years or older;
2. predominant disease: (*a*) bone, (*b*) other sites with or without bone;
3. previous radiation therapy: (*a*) 40% or less of bone marrow exposed ("limited"), (*b*) more than 40% of bone marrow exposed ("extensive").

After stratification, patients with "limited" radiation therapy were randomized between adriamycin, 60 mg/m² given in a single iv dose every 3 weeks × 2 and every 4 weeks thereafter, and the combination of 5-FU, 300 mg/m², and cyclophosphamide, 150 mg/m² administered iv every day for 5 consecutive days and repeated every 5 weeks from the first day of treatment. Patients with "extensive" prior radiation therapy initially received 40 mg/m² of adriamycin, 180 mg/m² of 5-FU, and 90 mg/m² of cyclophosphamide. Dosages were subsequently adjusted to keep the white blood cell (wbc) nadir above 2000 cells/mm³ and the platelet nadir above 75,000 platelets/mm³.

TABLE 1.—Percentage of bone marrow contained in various parts of the skeleton*

Anatomic area	Marrow volume (%)
Skull	13
Upper limb girdles	8
Sternum and ribs	10
Vertebrae	
Cervical	3
Thoracic	14
Lumbar	11
Sacrum	14
Pelvis (innominates plus femora, head and neck)	26

*Ref 6 (modified).

When a patient's disease progressed during initial therapy, he was crossed over to the other therapy if he met the eligibility criteria shown in figure 1.

Response to therapy was divided into two parts: actual tumor measurement (excluding prostate size) and assessment of ancillary factors (table 2). If actual palpable tumor was present, an objective response required a 50%

a. Biopsy-proven adenocarcinoma of the prostate which has metastasized and which is not controllable with routine ablative or additive hormonal therapy;

b. stage IV (T_{1-4}, N_x, $M_{1a \cdot 1b}$) disease with visible or measurable tumor present;

c. no prior treatment with an alkylating agent, 5-FU, or adriamycin;

d. no radiation therapy or chemotherapy within previous month;

e. no major surgery within the preceding 4 weeks;

f. performance status (Eastern Cooperative Oncology Group [ECOG] scale) < 4;

g. caloric intake of at least 1200 calories daily;

h. no evidence of active infection;

i. wbc at least 4100 cells/mm³ and platelets at least 130,000 platelets/mm³;

j. no evidence of active heart disease;

k. informed consent signed prior to the initiation of treatment;

l. serum creatinine not > 2.0.

FIGURE 1.—Eligibility criteria for entry in study.

TABLE 2.—Ancillary factors analyzed and ECOG performance score employed*

Ancillary factors	Better	Same	Worse
Pain	1	2	3
Weight change (≥5%)	1	2	3
Hemoglobin change (≥10%)	1	2	3
Performance score change (1 level)	1	2	3
Alkaline phosphatase change (≥25%)	1	2	3
Acid phosphatase change (≥25%)	1	2	3

*Overall score of 7 or less considered a response; overall score of 8–13 considered stable; overall score or more than 13 considered progression.

or greater reduction in tumor size with no new lesions appearing. Stable disease was defined as less than a 50% reduction, or less than a 25% increase, in tumor size. An objective progression required a 25% or greater increase in the size of a preexisting lesion or the appearance of a new lesion. If skeletal roentgenograms were used, a new area of sclerosis was considered a progression. If a bone scan was used, a new "hot spot" was considered progression. If only ancillary factors could be evaluated, a score of less than 8 was considered as a response, scores from 8 to 13 as stable, and a score of more than 13 as progression. Unless clearcut progression was noted, response to therapy was assessed 10 weeks after the initiation of therapy.

Table 3 outlines the tests used to observe the patients. A physical examination was performed prior to each treatment. During this examination patients were questioned about subjective changes experienced with therapy.

Between treatments, patients had a wbc count taken at least once a week and a platelet count once every other week.

RESULTS

Iliac crest bone marrow biopsies were performed in eight of the nine patients. Seven of these biopsies revealed metastatic tumor and one revealed fibrosis only. At the time of this procedure, sufficient blood was obtained in only one of the eight patients for an acid phosphatase determination. The bone marrow blood acid phosphatase was 38 units/liter with 52% tartrate inhibition vs a venous acid phosphatase level of 42 units/liter with 51% tartrate inhibition.

To date, nine patients have been entered in the study and eight are evaluable for response to therapy. Four patients received adriamycin at full dose as their initial treatment. Of these four patients, one with soft tissue metastases as his predominant disease had an objective response, but this lasted only 5 weeks and he subsequently died 12 weeks after initiation of chemotherapy. Of the other three patients, two have progressed (one subsequently failing 5-FU-cyclophosphamide therapy and one other stable on 5-FU-cyclophosphamide therapy at 5 weeks) and one remains stable at 5 weeks. Of the four patients initially treated with the combination of 5-FU and cyclophosphamide, three have progressed (one subsequently failing adriamycin therapy and two too ill for crossover) and one is stable at 10 weeks.

Toxicity

Most patients have experienced mild nausea with treatment, but no vomiting, diarrhea, or stomatitis has been noted. Only one wbc nadir below 2000 cells/mm^3 has been noted. No platelet nadirs below 75,000 platelets/mm^3 have been seen. Three patients have had their drug dose increased, one has had his drug dose decreased, and four have had no dosage change.

TABLE 3.—Schedule for followup laboratory tests

Tests	Test day												
Adriamycin:		21	42	70	98	126	154	172	200	228	256	284	312
5-FU + cyclophosphamide:	1		35	70	105	140	175	210	245	280	315	340	372
Wbc and platelet counts, hemoglobin	×	×	×	×	×	×	×	×	×	×	×	×	×
Chest X-rays	×		×	×	×	×		×		×			×
Bone marrow survey	×			×			×			×			×
Technetium-99 polyphosphate scan (bone)	×			×									
Venous acid phosphatase	×		×	×	×	×	×	×	×	×	×	×	×
Venous alkaline phosphatase	×		×	×	×	×	×	×	×	×	×	×	×
Bone marrow biopsy (iliac)	×			×									
Bone marrow acid phosphatase	×			×									
Excretory urogram	×			×				×					×
Creatinine	×			×		×		×		×			×
Bilirubin	×			×		×		×		×			×
Serum glutamic oxaloacetic transaminase	×			×		×		×		×			×
Urinalysis	×			×		×							

In general, toxicity has been mild and quite acceptable.

DISCUSSION

Our preliminary data have been reported. The drug doses and schedules we have used are quite tolerable. To date, only one of eight patients who received both treatment schedules has had what we would consider an objective response.

Assays of bone marrow serum acid phosphatase levels have not proved rewarding because the dense tumor replacement of normal marrow makes aspiration of sufficient blood for analyses frequently impossible.

The ancillary scoring system used seems to correlate reasonably well with actual tumor measurement by physical examination, bone roentgenograms, and bone scans. This system will undergo continued review.

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Clinical Trial of 2-Bromo-α-ergocryptine (NSC-169774) in Human Prostatic Cancer [1]

A. Coune and P. Smith [2,3,4]

SUMMARY

An EORTC type I clinical trial of 2-bromo-α-ergocryptine was performed in patients with prostatic carcinoma in clinical stages III and IV. Evaluating rules of response to the therapy were based on objective criteria. The maximum period of followup was 16 weeks. No objective remissions were observed in 24 patients, and in 13 of these patients, evidence of progression of the disease was apparent during the first 8 weeks of drug administration.

[Cancer Chemother Rep 59:209–213, 1975]

Because of the lethal cardiovascular side effects of estrogens (1) and the small number of other effective therapeutic procedures available for the treatment of prostatic carcinoma, it occurred to the EORTC Genito-Urinary Tract Cancer Cooperative Group B that clinical trials for assessing the possible value of several compounds in this cancer were urgently needed.

In most previous clinical trials of prostatic cancer, the duration of the survival time from the start of therapy had been used as a single objective parameter for evaluation of the response to therapy. Unfortunately, as shown by the Veterans' Administration Cooperative Urological Research Group (VACURG) studies, such a parameter may be biased by the occurrence of unknown side effects of the drug tested (1,2), as well as by the high incidence of lethal intercurrent diseases occurring in older men (as are most patients with prostatic cancer) (3). Moreover, such a parameter of evaluation was inadequate for screening studies such as the EORTC type I clinical trials.

PROTOCOL

A protocol with evaluating rules based on several objective criteria was designed. These objective criteria were of two types: specific and nonspecific. The former are parameters primarily related to the size of the prostatic tumor or its metastases and to the functional state of the tumor, while the latter are parameters whose value may be changed indirectly by modifications in the size of the tumor masses. The following parameters were taken into account as objective specific criteria: size of the prostatic tumor, number and size of soft-part

[1] 2-Bromo-α-ergocryptine: CAS reg. No. 22260-51-1; ergotaman-3',6',18-trione, 2-bromo-12'-hydroxy-2'-(1-methylethyl)-5'-(2-methylpropyl)-, (5'α)-, monomethanesulfonate (salt); CB-154.

[2] This study was done by the European Organization for Research on Treatment of Cancer (EORTC) Genito-Urinary Tract Cancer Cooperative Group B: chairman, *P. Smith;* secretary, *A. Coune;* representative of the EORTC Council, *E. Cooper;* and coordinator, *M. Staquet.* The following participated in this study: St. James's Hospital, Leeds, Great Britain (*P. Smith, V. G. McGirr, R. E. Williams,* and *G. J. Fellows*); Pontefract General Infirmary, Great Britain (*R. G. Robinson*); Institut Jules Bordet, Brussels, Belgium (*A. Coune* and *H. J. Tagnon*); and Hôpital Brugmann, Brussels, Belgium (*W. Grégoir* and *M. Chantrie*).

[3] The EORTC Genito-Urinary Tract Cancer Cooperative Group B is very grateful to Dr. Evans (Sandoz Ltd., Great Britain) and to Dr. Maeck (Sandoz Ltd., Belgium) for supplying 2-bromo-α-ergocryptine. The group is also much indebted to Dr. Maureen Harper who provided facilities for the assay of serum prolactin at the Tenovus Institute in Cardiff, Wales.

[4] *Reprint requests to:* A. Coune, MD, Institut Jules Bordet, Rue Héger-Bordet 1, 1000 Brussels, Belgium.

metastases (ie, superficial lymph nodes, cutaneous and subcutaneous nodules), number and size of osseous metastases and lung metastases, size of the liver (provided that hepatomegaly had been demonstrated to result from metastatic involvement), and value of the serum prostatic acid phosphatase. The following parameters were considered as objective nonspecific criteria: degree of dilatation of the upper urinary tract (only in patients without bladder-outlet obstruction), neurologic findings, value of the serum alkaline phosphatase, and performance status evaluated according to the Karnofsky scale (4). The subjective parameter of pain due to metastatic bone involvement was included in the criteria for evaluation because it was considered that no case of objective remission was compatible with the occurrence of intense bone pain.

Three types of response to therapy were considered: objective remission, status quo, and failure. Cases in which some tumor masses decreased in size while one or several other criteria indicated progression of the disease were evaluated as failures. These criteria were: progression of metastatic lesions other than the ones regressing, appearance of new metastatic lesions, increase in the serum prostatic acid phosphatase level, increase in the serum alkaline phosphatase level (unless this increase was due to recalcification of previous lytic bone lesions), appearance of hypofibrinogenemia, and occurrence of intense bone pain. Patients who died before the followup period was completed were considered failures. The appearance of severe side effects from the therapy, endangering the life of the patient, required the discontinuation of the drug; such a case had to be classified as a failure.

Due to the lack of accuracy of the rectal examination, to the rather infrequent occurrence of peripheral metastatic nodules and lymph nodes, and to the most frequent type of osseous metastases (plastic), direct evidence of an objective remission may be difficult to prove in some cases of prostatic carcinoma. Therefore, some arbitrary rules of evaluation were elaborated allowing a clear response to the several types of clinical pictures observed in patients suffering from prostatic carcinoma. These rules specify the type of improvement of the objective specific criteria needed as evidence for an objective remission; all of them require at least status quo of the objective nonspecific criteria.

Before the therapy was started a detailed workup was carried out; this workup was repeated at the end of an 8-week followup period, allowing a first evaluation of each case. If clearcut progression was evident, administration of the compound was discontinued. On the other hand, if a remission or a status quo was observed, the compound was given for another 8-week period. At the end of this second period, a final evaluation of the case was performed. Great care was taken to avoid intake by patients admitted to the study of other drugs, eg, tranquilizers, psychomotor stimulants, or α-methyldopa, all of which are known to interfere with the secretion of prolactin.

CLINICAL TRIAL

Because it appeared from in vitro and in vivo studies in animals that prolactin might play a role in controlling the activity of the prostate (5,6), perhaps by amplifying the effectiveness of androgen in several ways, the EORTC Group launched a type I clinical trial to test the possible value in human prostatic carcinoma of an ergocornine derivative, 2-bromo-α-ergocryptine, a well-known effective inhibitor of prolactin secretion in man (7,8). The compound was taken orally, three times a day at meal time; the daily dosage was 7.5 mg. Twenty-eight patients, clinical stages III and IV according to the VACURG classification (1), were admitted to the study; 20 of these had received no previous endocrine therapy while eight patients were estrogen resistant. As indicated in table 1, several side effects were observed, the most frequent and disturbing being nausea and vomiting. This side effect occurred in 11 patients, and although it disappeared within a few days after the start of the therapy in most patients, discontinuation of the compound was necessary in three patients. One patient, having experienced fainting after the start of the therapy, refused to continue taking the drug. Edema was observed in two patients, dizziness was noted by one patient, and in another two

patients, slight transient hyperbilirubinemia was observed. No correlation between the incidence of these side effects and the clinical stage of the prostatic carcinoma was apparent. No hematologic toxicity and no hypercalcemia were observed. Because the drug was stopped in four patients, the cases of only 24 were evaluated. Their ages ranged from 54 to 88 years with a median age of 71 years.

TABLE 1.—Toxicity from 2-bromo-α-ergocryptine therapy in 28 patients with prostatic carcinoma

Toxic effect	No. of patients
Nausea, vomiting	11 (drug discontinued in 3)
Diarrhea	0
Skin changes, hair loss	1 (photosensitivity occurred while another drug was being given at the same time)
Mucous membrane toxicity	0
Dizziness	1
Fainting	1 (drug discontinued)
Edema	2
Hyperbilirubinemia	2
Hematologic toxicity	0
Hypercalcemia	0

As indicated in table 2, there were seven estrogen-resistant patients and six clinical stage III patients. The distribution of the metastatic parameters in the 18 stage IV patients is recorded in table 3. All estrogen-resistant patients had an increased serum prostatic acid phosphatase activity as well as metastatic bone involvement, while less than 50% of the patients who had not previously received hormonal treatment had an elevated serum prostatic acid phosphatase level. Eleven of these 13 patients had osseous metastases. Anemia (hemoglobin level < 14 g/100 ml) was present in all estrogen-resistant clinical stage IV patients while it occurred in nine of the 13 other patients. Increased serum urea (>45 mg/100 ml) was observed in 33% of the patients.

RESULTS

No objective remissions were observed among the 24 evaluable patients. At the end of the 16-

TABLE 2.—Distribution of the 24 evaluable patients

Clinical stage	No. of patients		
	No previous endocrine treatment	Estrogen resistant	Total
III	4	2	6
IV	13	5	18
Total	17	7	24

TABLE 3.—Distribution of metastatic parameters in 18 clinical stage IV patients

	No. of patients with—			
	Soft tissue metastases	Osseous metastases	Visceral metastases	Increased serum prostatic acid phosphatase activity
Patients with no previous endocrine treatment (13 patients)	4	11	2	5
Estrogen-resistant patients (5 patients)	1	5	2	5

week followup period five patients, all of whom were in clinical stage IV, had died. Three of these deaths occurred during the first 8 weeks of drug administration. Only two patients showed no modification of the parameters of evaluation at the end of the 16-week followup period. All other patients showed, at that time or after the first 8-week period of followup, signs of disease progression. An increase in the size of the prostatic tumor was noted in eight patients, appearance of new bone lesions in six, appearance of new soft tissue lesions (lymph nodes) in two, and an increase in serum prostatic acid phosphatase activity from a normal to an abnormal level in three and from an abnormal pretreatment level to a still higher level in another five. Hematuria occurred in two patients and hydronephrosis developed in one patient. Three patients jumped from clinical stage III to clinical stage IV during administration of 2-bromo-α-ergocryptine. In five patients the progression of the disease was indicated by the modification of only a single parameter: an increase in the size of the prostatic tumor in one patient, the appearance of new bone lesions in three patients, and an increase in the serum prostatic acid phosphatase level in one patient.

In 13 patients, the final evaluation was completed at the end of the first 8-week period of followup; three of these patients had died and four had shown signs of progression of the disease before the end of the 8-week period. During the next 8-week followup period two more patients died and seven showed signs of progression.

Serum prolactin levels were measured in 11 patients before and during 2-bromo-α-ergocryptine treatment. The blood was taken at 10 AM, in most cases on 2 consecutive days. The prolactin was measured by a radioimmunoassay performed at the Tenovus Institute at Cardiff, Wales. Table 4 indicates the measured values; in most cases there was a decrease from the pretreatment level. In one patient an unexplained increase of the prolactin level was noted.

TABLE 4.—Serum prolactin levels*

Patient No.	Serum prolactin levels (milliunits/ml)— Before drug treatment	During drug treatment
1	0.18	0.06
2	0.16	0.05
3	0.15	0.09
4	0.13	0.08
5	0.11	0.05
6	0.10	0.22
7	0.08	0.05
8	0.07	0.01
9	0.06	0.02
10	0.05	0.02
11	0.05	0.05

*Standard: Medical Research Council research standard A.71-222. Antibody: raised in rabbits to human amniotic fluid.

DISCUSSION

In this clinical trial, 2-bromo-α-ergocryptine failed to induce remission of prostatic carcinoma in 24 patients (clinical stages III and IV). Followup of the patients for a 16-week period was sufficient to demonstrate the absence of activity of the compound. Since status quo of the lesions was observed in only two patients who completed the trial, possible acceleration of tumor growth by the compound may be considered. However, comparison of the estrogen-resistant patients' status before and during administration of 2-bromo-α-ergocryptine does not seem to validate this hypothesis. On the other hand, it would be interesting to add this drug to estrogens in patients not responding or no longer responding to this endocrine therapy. In such cases, preventing the increase in prolactin secretion induced by estrogens by the addition of 2-bromo-α-ergocryptine might perhaps result in a higher remission rate and a longer remission duration.

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Comparison of Adriamycin (NSC-123127) and 5-Fluorouracil (NSC-19893) in Advanced Prostatic Cancer [1]

William D. DeWys [2,3]

Cytotoxic chemotherapy for adenocarcinoma of the prostate has received limited systematic evaluation. When this study was planned, cumulated data in the literature included: nine responses to 5-fluorouracil (5-FU) out of 24 evaluable patients (37%); [4] three responses to adriamycin out of 14 evaluable patients (23%); [5] and a response rate of about 25% for several alkylating agents.[4] From these data we selected 5-FU and adriamycin for a comparative phase II and III study. Between December 1, 1973, and April 18, 1974, 26 patients were entered into this study and a preliminary assessment of their response to therapy provides the basis for this report.

MATERIALS AND METHODS

Patients were selected for this study if they had histologically confirmed adenocarcinoma of the prostate not amenable to curative surgical treatment or radiotherapy. All patients had previously received ablative or additive hormonal therapy and had either relapsed during this therapy or failed to respond to it. At least 6 weeks had elapsed after the initiation of the hormonal therapy. All patients had either a measurable tumor or an elevated acid phosphatase level or both which could be followed to assess tumor response. Patients were required to have adequate hepatic, renal, and marrow functions (bilirubin <1.5 mg/100 ml, serum glutamic oxaloacetic transaminase <80 IU, blood urea nitrogen <30 mg/100 ml, creatinine <1.5 mg/100 ml, white blood cell count >4000 cells/mm^3, and platelet count >100,000 platelets/mm^3). All patients gave written informed consent.

Before randomization, the patients were stratified by variables which (*a*) might be prognostically important, (*b*) might influence a patient's ability to tolerate full doses of chemotherapy, and (*c*) were the end points of patient evaluation. Each patient's general status was assessed using the Eastern Cooperative Oncology Group performance status criteria. Patients were then stratified according to performance status: 0–1 (normal or slightly limited activity) or 2–3 (moderate to severely limited activity). Because the presence of bony metastases has been shown to influence prognosis, patients were stratified as to the presence or absence of demonstrable bony metastases.[6] Patients were classed as to the amount of previous radiotherapy they had received (limited or extensive) because the extent of previous radiotherapy might determine the dose of chemotherapy that could be tolerated. Because measurable tumor (soft tissue or lung nodules, well-defined retroperitoneal lymph nodes [defects on intravenous pyelogram], or hepatic enlargement >5 cm below the costal margin) or abnormal acid phosphatase were the two end points for evaluation of tumor response, patients were stratified according to the presence or absence of these two parameters. Patients were randomized to receive either adriamycin or 5-FU. Patients progressing at 6 weeks or failing to respond (no change) by 12 weeks were crossed over to the other treatment.

[1] 5-Fluorouracil: CAS reg. No. 51-21-8; 5-FU. Adriamycin and 5-FU were supplied by Cancer Therapy Evaluation, Division of Cancer Treatment, National Cancer Institute.

[2] Medical Oncology Section, Department of Medicine, Northwestern University Medical School, Chicago, Ill. Member of the Eastern Cooperative Oncology Group.

[3] *Reprint requests to:* William D. DeWys, MD, Medical Oncology Section, Department of Medicine, Northwestern University Medical School, Chicago, Ill 60611.

[4] Slavik M, and Carter SK. Single agent prostatic cancer, a quick review. Personal communication, 1973.

[5] Carter SK. Personal communication, Mar 1973.

[6] Huse R, Byar DP, Bailar JC, et al. A multi-variant exponential model for predicting survival of prostatic cancer patients with time censored data. Personal communication.

Patients who had received no previous radiotherapy or limited radiotherapy received either 5-FU (600 mg/m²/week) intravenously (iv) or adriamycin (60 mg/m² every 3 weeks) iv. Patients who had received extensive prior radiotherapy were initially treated with reduced doses: 5-FU (400 mg/m²/week iv) or adriamycin (40 mg/m² every 3 weeks iv). If after 6 weeks at these reduced doses the patients had not experienced significant hematologic toxicity, the dose of either drug was escalated by 50%. Response of measurable disease was categorized using the following definitions:

complete response—complete disappearance of all measurable disease with no new lesions developing;

partial response—a 50% reduction in the sum of the products of the horizontal and vertical tumor diameters;

no change—tumor shrinkage not fulfilling the partial response criteria or an increase in tumor size ($\leq$ a 25% increase in the sum of the products of the horizontal and vertical tumor diameters);

progression—a greater than 25% increase in the sum of the products of two tumor diameters or the development of new lesions.

For patients without measurable disease but with an elevated acid phosphatase level, a reduction by 50% in the pretreatment acid phosphatase level was considered a partial response. A drop of lesser magnitude or an increase of less than 50% was considered no change, and a greater than 50% increase was considered disease progression.

RESULTS

The results of treatment are shown in table 1. Of 14 evaluable patients treated with 5-FU, five fulfilled the criteria for a partial response (36%). The patients showing a partial response included three who showed a greater than 50% reduction in their acid phosphatase level, one who showed a greater than 50% shrinkage of tumor, and one who showed simultaneous reduction of greater than 50% in tumor size and a lowered acid phosphatase level. Of eight evaluable patients treated with adriamycin, one showed a partial response as measured by reduction in liver size. Too few patients have entered the crossover phase to draw significant conclusions, but there has been one response in each of the crossover groups.

The toxicity response has not been completely evaluated, but to date none of the patients have experienced severe toxicity.

DISCUSSION

It must be emphasized that these are very preliminary observations which permit no conclusions as to the comparative efficacy of these two treatments. The response rate observed for 5-FU is essentially similar to that compiled from the literature.[7] The observed frequency of response to adriamycin is less than that observed for prostatic cancer by some investigators, but this difference may not be real due to the small number of patients studied in this treatment arm. One may, however, question the adequacy of the dose of adriamycin used in the present study. The Southwest Oncology Group has been comparing different doses of adriamycin and their data for prostatic cancer are presented in table 2.[8] One may note that doses of 60 mg/m² or higher yield significant response rates, whereas doses lower than this had almost no effect. We intend to analyze our data in more detail to determine how many patients were treated at the lower initial dose (patients who had received extensive radiotherapy) or to see whether patients failing to respond had significant downward modifications of their drug doses during the course of therapy.

We plan to continue this study so that an adequate number of patients will be treated to permit a statistically significant comparison of response rates for the two treatments. We also plan to compare the survival results of these two treatments and to correlate response and survival time. The crossover design will permit an evaluation of sequential treatment using these two agents and an evaluation of possible cross resistance. In patients having both measurable tumor and elevated acid phosphatase levels, serial studies should permit the comparison of these two parameters as a measure of drug effect.

[7] *See* footnote 4.

[8] O'Bryan RM. Personal communication, 1974.

TABLE 1.—Response of prostatic cancer to adriamycin and 5-FU*

Drug	No. of patients			
	Partial response	No change	Progression	Non-evaluable
5-FU	5	7	2	3
Adriamycin	1	5	2	1

*From Eastern Cooperative Oncology Group study No. 2273.

TABLE 2.—Response of prostatic cancer to adriamycin*

Dose (mg/m^2)	No. of patients responding/total No. of evaluable patients	Response (%)	
Good risk			
75	3/9	33	38
60	2/4	50	
45	0/6	0	6
Poor risk			
50	1/6	17	
25	0/6	0	

*From Southwest Oncology Group study No. 7206.

Oral Estramustine Phosphate (NSC-89199) in the Treatment of Advanced (Stage D) Carcinoma of the Prostate[1]

A. Mittelman, S. K. Shukla, K. Welvaart, and G. P. Murphy[3,4]

SUMMARY

Thirty-two patients with stage D carcinoma of the prostate were treated with oral estramustine phosphate at a dose of 15 mg/kg/day from 3 to 15 months. Objective remissions, reduction of greater than 50% of measurable lesions such as soft tissue masses, lymph nodes, and prostatic masses, were seen in seven of 32 patients (22% response rate). Subjective response, ie, relief of pain, weight gain, sense of well being, and improved performance status, occurred in all objective responders and in seven other patients with stable disease (15 of 32 patients = 47%). No hematologic, hepatic, or renal toxic effects were observed. Transient nausea occurred early in one half of the patients and nausea and vomiting was dose limiting in only two patients. Oral estramustine phosphate is well tolerated and worthy of further clinical use.

[Cancer Chemother Rep 59:219–223, 1975]

Estramustine phosphate (fig 1) was first used by Swedish and other European investigators for advanced carcinoma of the prostate (1,2). While clinically effective, the intravenous form of estramustine phosphate had a high incidence of regional toxicity (3). For this reason an oral preparation was developed. We have recently reported a phase I study using oral estramustine phosphate in advanced hormonally resistant carcinoma of the prostate (4).[5] This study demonstrated that a dose of 15 mg/kg/day given in three divided doses was therapeutically effective and well tolerated. This report involves

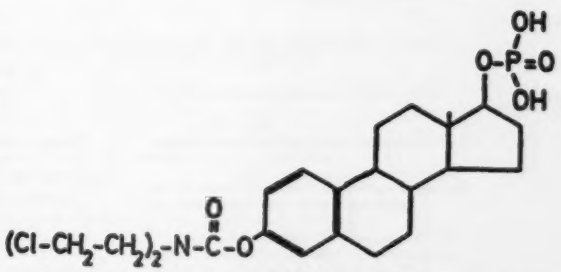

FIGURE 1.—Estramustine phosphate (Estracyt), a new agent under current evaluation in advanced prostatic carcinoma.

[1] Estramustine phosphate: CAS reg. No. 4891-15-0; estra-1,3,5(10)-triene-3,17β-diol, 3-[bis(2-chloroethyl)-carbamate]17-(dihydrogen phosphate); Estracyt.

[2] Supported in part by grant RR-262 from the General Clinical Research Centers Program of the Division of Research Resources, National Institutes of Health, Department of Health, Education, and Welfare.

[3] General Clinical Research Center, Department of Surgery, Roswell Park Memorial Institute, NY State Department of Health, Buffalo.

[4] *Reprint requests to:* A. Mittelman, MD, General Clinical Research Center, Department of Surgery, Roswell Park Memorial Institute, NY State Department of Health, 666 Elm St, Buffalo, NY 14203.

[5] Murphy GP, Sandberg AA, Muntzing J, et al. A clinical pharmacologic study of oral estramustine phosphate (Estracyt) in patients with adenocarcinoma of the prostate. Presented at the Conference of the International Society of Urology. Amsterdam, Netherlands, July, 1973.

32 patients who received oral estramustine phosphate at the stipulated dose.

Patient Study Group—Entry Criteria

All of the 32 patients entered into this study had histologically documented carcinoma of the prostate and had: (*a*) progressive stage D disease; (*b*) no response to estrogen therapy and castration; (*c*) measurable or evaluable disease.

A 2-week or more interval must have elapsed between any previous therapy and the administration of oral estramustine phosphate.

Schedule of Drug Administration

All patients received a dose of 15 mg/kg/day of estramustine phosphate in three divided doses. Occasionally, small doses of phenothiazines were used to overcome the transient nausea experienced by most patients when they initially received the drug. To achieve a satisfactory oral intake of this drug most patients were admitted to the hospital for several days. Metabolic studies could also be conveniently carried out at this time. Patients were seen in the outpatient clinic every 2 weeks whenever possible. Patients who lived several hundred miles away were seen at 6–8-week intervals and were usually admitted for several days of study. A complete blood cell count and differential, hepatic and renal function studies, and acid and alkaline phosphatase levels were determined at each clinical visit. Roentgenographic studies including intravenous pyelograms were performed as indicated. Bone scans were regularly performed for all clinical patients.

RESULTS

Response

The overall objective and subjective response rate is given in table 1. Table 2 gives a more detailed analysis of the nature of the objective response and its duration for the eight responders. Table 3 indicates certain aspects of the natural history of carcinoma of the prostate in the responders. No correlation could be found between objective response and prior response to estrogen therapy or orchiectomy. The age range of responders covered a 20-year span. No correlation could be seen between duration of disease prior to estramustine phosphate therapy and rate of response after administration of estramustine phosphate.

TABLE 1.—Oral estramustine phosphate in stage D carcinoma of the prostate

No. of evaluable patients	Dose schedule	No. of responses/%	
		Objective	Subjective
32	15 mg/kg/day in 3 divided doses	7/25	15/47

TABLE 2.—Analysis of objective responses to estramustine phosphate (15 mg/m²/day)

Patient No.	Onset of response after initial dose (mos)	Type of response	Duration of response (mos)
1	1	Acid phosphatase level, 17–4.0; prostatic nodules completely regressed	6+ Still receiving therapy
2	1.5	Decrease in size of primary malignancy; some improvement in osteoblastic metastases	12+ Still receiving therapy
3	1	Return of elevated acid and alkaline phosphatase values to normal	4 Died of myocardial infarct
4	1.5	Soft tissue mass and edema of left leg completely regressed; decrease in acid phosphatase level to normal	6 Died of progressive disease

TABLE 2.—Analysis of objective responses to estramustine phosphate (15 mg/m²/day)—Continued

Patient No.	Onset of response after initial dose (mos)	Type of response	Duration of response (mos)
5	2	Acid and alkaline phosphatase level returned to normal; improvement in left hydronephrosis	16+ Still receiving therapy
6	2	Decrease in size of prostatic mass	6 Died of progressive disease
7	1.5	Decrease in size of prostatic primary and pelvic node	9+ Off study because of gastrointestinal toxic effects
8	1	Possible objective response; decrease in acid phosphatase level	1+ Too early to evaluate

TABLE 3.—Relationship of previous endocrine therapy to response to estramustine phosphate

Type of response	No. of patients	Response to prior estrogen therapy or orchiectomy	
		No	Some
Objective	7	4	4
Subjective	32	15	17

The type of objective response that was seen was confined to regression of soft tissue or prostatic masses, and a return of elevated serum acid and alkaline phosphatase values to normal. We have not seen a significant or consistent improvement in osteoblastic lesions. Osteolytic lesions were not observed in the objective responders' group. Among the responders' group, three patients have been in remission for more than 1 year. If a patient responded to estramustine phosphate, he did so within 2 months of initially receiving therapy.

Subjective Response

Table 4 lists the 15 subjective responders and details the analysis of their response (fig 2). All objective responders had impressive subjective responses. More than one half of the responders achieved benefit for 6 months or more, one third had transient benefit (less than 4 months), and the remainder of the patients are still in the early stages of their therapy. Pain relief and concomitant improvement in

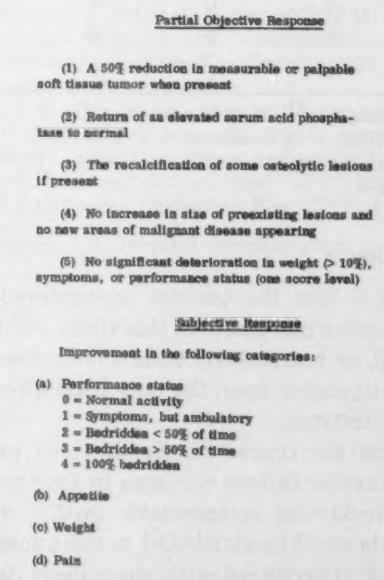
Partial Objective Response
(1) A 50% reduction in measurable or palpable soft tissue tumor when present
(2) Return of an elevated serum acid phosphatase to normal
(3) The recalcification of some osteolytic lesions if present
(4) No increase in size of preexisting lesions and no new areas of malignant disease appearing
(5) No significant deterioration in weight (> 10%), symptoms, or performance status (one score level)
Subjective Response
Improvement in the following categories:
(a) Performance status
0 = Normal activity
1 = Symptoms, but ambulatory
2 = Bedridden < 50% of time
3 = Bedridden > 50% of time
4 = 100% bedridden
(b) Appetite
(c) Weight
(d) Pain

FIGURE 2.—Evaluation of therapy—National Prostatic Cancer Project criteria. Prepared by the National Prostatic Cancer Project (Gerald P. Murphy, Director).

performance status were impressive. Two totally bedridden patients became ambulatory. In this small group it is not possible to correlate performance status with objective or subjective response. It is true though that those patients with a good initial performance status have been among the greater-than-1-year survivors.

TABLE 4.—Subjective response to oral estramustine phosphate*

	Subjective responses—								
	Before estramustine phosphate				After estramustine phosphate				
Patient No.	Pain†	Appetite‡	Weight‡	Performance status§	Pain	Appetite	Weight	Performance status	Duration (mos)
1	0	0	0	0	0	+	+	0	15+
2	+	0	0	1	0	+	+	0	15+
3	++	–	–	2	0	+	+	0	10
4	+	0	0	1	0	+	+	0	10+
5	++	–	–	4	+	+	+	2	2
6	+	0	0	4	0	+	0	1	6
7	++	–	–	2	0	+	+	1	9
8	+	0	0	1	0	+	0	0	7
9	++	–	–	3	+	+	+	1	1
10	++	0	0	2	+	+	+	1	1
11	+	0	0	1	0	+	0	1	2
12	+	–	–	1	0	+	+	1	4
13	++	0	0	3	0	+	+	1	3
14	+	0	0	1	0	+	+	0	2+
15	+	0	0	2	0	+	+	0	3+

*Response rate = 15 of 32 patients (47%).
†0 = None; + = moderate; ++ = severe.
‡0 = No change; – = decrease.
§*See* fig 2.

Toxic Effects

Table 5 lists the toxicity encountered with estramustine phosphate in this study. No hepatic, renal, or hematologic toxic effects were seen at the stipulated dose; the only toxic effect was gastrointestinal.

During the course of the previous phase I study, cardiac failure was seen in four patients with preexisting symptomatic cardiac disease (4). This could be attributed to the nausea and vomiting experienced with subsequent deletion of oral diuretics and cardiotoxic drugs. No instances of cardiac failure, edema, or water retention were seen in the current study.

TABLE 5.—Toxic effects of estramustine phosphate*

Toxic effects	No. of patients with toxic reactions/ total No. of patients
Hepatic	0/32
Renal	0/32
Hematologic	0/32
Gastrointestinal	
Early†	15/32
Late‡	2/32

*15 mg/kg/day in 3 divided doses.
†Transient nausea and occasional vomiting lasting 1 wk; readily controlled with phenothiazines.
‡Intractable nausea and vomiting occurring 6–8 wks after initiation of therapy; therapy was then discontinued.

DISCUSSION

Oral estramustine phosphate has modest but significant activity in patients who are not responsive to hormonal manipulation. The drug is well tolerated and its toxicity does not involve any major organ system. It may be particularly useful for those patients who cannot receive myelosuppressive chemotherapeutic agents because of poor bone marrow reserve. Long-term therapy for well over a year for three of our patients has revealed no cumulative toxic effects of any type except for the occasional patient who exhibits gastrointestinal intolerance after 1 or 2 months. Clinically significant water retention has not been seen. Only one patient has had any feminizing effects such as breast tenderness.

The mechanism of action of estramustine phosphate in man is not clear. It was hoped that the steroid moiety would direct the alkylating moiety to the susceptible cell. Intracellular hydrolysis would then release the alkylating agent, thus achieving selectivity but no systemic toxicity.[6] We can safely say that there is no systemic toxicity that can be attributed to the alkylating moiety. Whether the drug exerts its beneficial effect in the proposed fashion is not known. We do not believe that estramustine phosphate is a slow-release form of estrogen and thereby has whatever benefit it produces as estradiol. There is little evidence for significant clinical estrogen effect, and among our responders are those who either failed to respond to estrogen or no longer respond. The experimental data of Muntzing and colleagues indicate that rodents and dogs can split estramustine phosphate into a biologically active steroid and an alkylating agent (5). Metabolic experiments in man and primates indicate a very different pattern of the metabolism of estramustine phosphate. In man, 75%–80% of the administered doubly labeled radioactive drug cannot be accounted for.[7] The value of oral estramustine phosphate primary therapy, ie, before hormonal manipulation, has not been explored by us. Should current clinical trials confirm the activity of oral estramustine phosphate, such a program would be justified.

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The Veterans' Administration Cooperative Urological Research Group Studies of Carcinoma of the Prostate: A Review [1]

Clyde E. Blackard [2,3,4]

SUMMARY

The Veterans' Administration Cooperative Urological Research Group has conducted three consecutive randomized clinical trials of various treatments for all stages of prostatic carcinoma. Thus far, a total of 3774 patients have been entered into these three main studies. The first study showed that early endocrine treatment of patients with advanced prostatic cancer did not increase overall survival when compared to initial treatment with placebo alone. Diethylstilbestrol, when given in a dose of 5.0 mg/day, was associated with an increased incidence of cardiovascular deaths. In the second study, diethylstilbestrol given in a dose of 1.0 mg/day has been as effective as the 5.0-mg dose in controlling the prostatic carcinoma but has been associated with a lower incidence of cardiovascular deaths. Patients are still being entered into the third study and it is too early to report the findings in detail.

[Cancer Chemother Rep 59:225–227, 1975]

In March of 1960, 14 Veterans' Administration Hospitals began a prospective randomized clinical trial of various treatments for all stages of prostatic carcinoma. Patients with stage I or II were randomized between radical prostatectomy plus placebo and radical prostatectomy plus diethylstilbestrol (DES),[5] 5 mg/day. Patients with stage III or IV were randomized between placebo, DES (5 mg/day), orchiectomy plus placebo, and orchiectomy plus DES (5 mg/day). All patients had histologically proven and previously untreated prostatic cancer. The

[1] Supported by Public Health Service grant CA-12443 from the National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[2] Veterans' Administration Hospital (VAH), Minneapolis, Minn.

[3] The following members in the Prostate Studies I and II and the Focal Carcinoma Study of the Veterans' Administration Cooperative Urological Research Group participated in this study: *Earl Haltiwanger*, VAH, Atlanta, Ga; *Paul W. Gonick*, VAH, Bronx, NY; *Lino J. Arduino*, Veterans' Administration Center (VAC), Des Moines, Iowa; *Maxwell Malament* (deceased), VAH, East Orange, NJ; *Howard C. Kramer*, VAH, Baltimore, Md; *Arthur J. Bischoff*, VAH, Long Beach, Calif; *Joseph Jorgens*, VAC, Los Angeles, Calif; *Henry I. Berman*, VAH, Louisville, Ky; *Paul O. Madsen*, VAH, Madison, Wis; *Clyde E. Blackard, Donald Gleason, U.S. Seal*, and *Richard P. Doe*, VAH, Minneapolis, Minn; *George T. Mellinger*, VAH, Kansas City, Mo (chairman, Prostate Studies I and II); *James S. Elliot*, Palo Alto, Calif; *Leslie E. Becker*, VAC, Leavenworth, Kan; *William L. Parry*, VAH, Oklahoma City, Okla; *Willis P. Jordan*, VAH, Memphis, Tenn; *Francis F. Bartone*, VAH, Omaha, Neb; *Prince Beach*, VAH, Houston, Tex; *Venancio R. Quiambao*, VAH, Cincinnati, Ohio; *Robert B. Higgins*, VAH, Portland, Ore; *A. Hardy Ulm III* (deceased), VAH, New York, NY; *Lloyd S. Rogers*, VAH, Syracuse, NY; *Raymond Yesner*, VAH, West Haven, Conn; *F. K. Mostofi*, Armed Forces Institute of Pathology, Washington, DC; *Lyndon E. Lee*, Veterans' Administration Central Office, Washington, DC; *John C. Bailar III* and *David P. Byar*, NCI, Bethesda, Md; and the VAH, Lake City, Fla.

[4] *Reprint requests to:* Clyde E. Blackard, MD, Veterans' Administration Hospital, 54th St and 48th Ave, Minneapolis, Minn 55417.

[5] Diethylstilbestrol: NSC-3070; 4,4'-stilbenediol, *α,α'*-diethyl-; stilbestrol. Supplied by Cancer Therapy Evaluation, Division of Cancer Treatment, NCI.

protocol specified that an assigned treatment could be changed whenever the investigator felt this was necessary for the patient's welfare. A total of 2314 patients were entered into the first study which was continued until March of 1967 and the results were initially reported in 1967 and updated in 1973 (1,2). Some of the important findings of this study will be mentioned. The Veterans' Administration Cooperative Urological Research Group (VACURG) concluded that early endocrine treatment of stage III and IV patients did not increase their overall survival when compared to initial treatment with placebo alone. Fewer deaths from prostatic cancer were observed in stage III and IV patients receiving one of the two treatments containing DES as their initial treatment but this advantage was offset by an increase in cardiovascular deaths. Cardiovascular deaths in estrogen-treated patients occurred predominantly during the first year of treatment. Consequently, the VACURG suggested that patients with advanced prostatic cancer not be treated with hormones until the cancer caused symptoms. By withholding hormonal treatment until the patient became symptomatic, survival rates were not adversely affected (3).

Between April 1967 and May 1969, a second VACURG study was conducted. Treatments for stage I and II were randomized between radical prostatectomy plus placebo and placebo alone. Patients with stage III or IV were randomized between placebo and 0.2, 1, or 5 mg/day of DES. A total of 561 patients were entered into the second study and the results were published in 1970 (4). In terms of survival, placebo was as good as 1 mg of DES in treating stage III patients, but was significantly worse than 1 mg of DES in treating stage IV patients. Thus far, the 1-mg dose of DES has been as effective as the 5-mg dose of DES in controlling the prostatic cancer in both stages but has been associated with a lower incidence of cardiovascular deaths.

A third VACURG study has been in progress since June 1969. Treatments for stage I and II patients were the same as for the second study. Treatments for stage III and IV patients were randomized between 1 mg/day of DES, 1.25 mg/day of Premarin for 1 month followed by 2.5 mg/day, 10 mg of medroxyprogesterone acetate three times daily, and 1 mg/day of DES plus 10 mg of medroxyprogesterone acetate three times daily.[6] Thus far, 899 patients have been entered into the third study. Up to the present time, it does not appear that Premarin, medroxyprogesterone acetate, or the combination of DES and medroxyprogesterone acetate is any better than DES alone in terms of survival or effects on the cancer.

In 1972, Byar and the VACURG (5) reported a study of 148 stage I patients who were not entered into the main study either because they were too old or too ill for radical prostatectomy or because they refused radical prostatectomy. This group was entered into a separate clinical trial called the Focal Carcinoma Study. In this study, treatments were randomized between placebo, 5 mg/day of DES, orchiectomy plus placebo, and orchiectomy plus 5 mg/day of DES. Patients were entered into the Focal Carcinoma Study between July 1964 and April 1969. Presently, there has been only one death due to prostatic cancer in this group. When the Focal Carcinoma Group was compared to a group of 120 stage I patients who had had radical prostatectomy from the first study (adjustments were made for age differences), the difference in survival between the two groups was not significant at any time. These findings suggested that radical prostatectomy may not be indicated for most patients with stage I carcinoma.

Presently, 45 scientific papers pertaining to clinical and laboratory studies of prostatic cancer have been published by the VACURG. Additional clinical and laboratory research is currently being conducted by members of this Group. For example, Dr. Gleason at the Minneapolis Veterans' Administration Hospital has developed a method of predicting the prognosis of prostatic cancer patients from the appearance of the primary and secondary histologic patterns of the tumor (6). Dr. Hovsepian at the Palo Alto Veterans' Administration Hospital

[6] Medroxyprogesterone acetate: NSC-26386; CAS reg. No. 71-58-9; pregn-4-ene-3,20-dione, 17-hydroxy-6α-methyl-, acetate; Provera.

Premarin: conjugated estrogenic hormones.

has developed a method of measuring the pattern and area of osseous metastases and has correlated this with survival. There have also been several reports of biochemical studies conducted in our reference laboratory on sera obtained from prostatic cancer patients before and after treatment. These have included biochemical studies of serum acid phosphatase (7), testosterone (8), corticosteroid-binding globulin (9), and total and free cortisol (10). Current laboratory studies are concerned with blood coagulation, serum and tissue zinc concentrations, serum cholesterol and triglycerides, and fibrinogen and plasminogen levels.

A fourth VACURG study of prostatic carcinoma is being planned.

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Clinical Results With Estramustine Phosphate (NSC-89199): A Comparison of the Intravenous and Oral Preparations [1]

Torgny Nilsson and Gösta Jönsson [2,3]

SUMMARY

The clinical results with estramustine phosphate in far-advanced carcinoma of the prostate refractory to other treatments are presented. Good clinical results have been achieved in about 40% of the 90 patients treated and regression of metastases has occurred in about 20%. Estramustine phosphate in the primary treatment of far-advanced carcinoma of the prostate shows very good and promising clinical results. A total of 132 patients were treated.

[Cancer Chemother Rep 59:229–232, 1975]

Estramustine phosphate (fig 1) has been used at the Department of Urology, University of Lund, Sweden, since 1967 in an intravenous (iv) preparation and since 1971 in an oral preparation. As is shown in figure 2, we have been using the oral compound more frequently.

The following criteria were required to start estramustine phosphate therapy:

1. prostatic carcinoma not responding to conventional therapy;
2. far-advanced tumor rapidly growing and most often undifferentiated; and
3. a life expectancy of less than 6 months.

The dosage has been the following: 300–450 mg/day for the iv preparation and 600–900 mg/day for the oral preparation. Some patients were given estramustine phosphate at once because of the aggressiveness of the disease. The patients are thus divided into two groups: those in whom the tumor has not responded to previous therapy and those treated with estramustine phosphate as primary therapy. All together, 132 patients were treated for more than 6 months and 90 of them did not respond

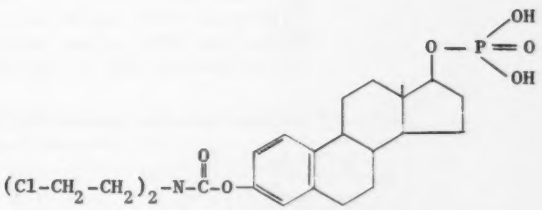

FIGURE 1.—Chemical structure of estramustine phosphate.

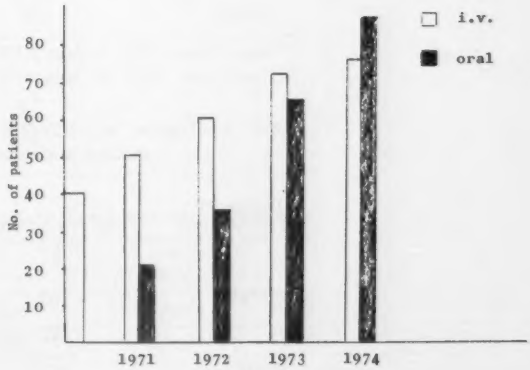

Fig. 2

FIGURE 2.—No. of patients treated with estramustine phosphate.

[1] Estramustine phosphate: CAS reg. No. 4891-15-0; estra-1,3,5(10)-triene-3,17β-diol, 3-[bis(2-chloroethyl)-carbamate] 17-(dihydrogen phosphate): Estracyt.

[2] Department of Urology, University of Lund, Sweden.

[3] *Reprint requests to*: Torgny Nilsson, MD, DSc, Department of Urology, University of Lund, Sweden.

to the previous therapy. We will primarily discuss these patients in this paper.

The clinical response is defined as good, uncertain, or none (table 1). Uncertain effect indicates no progression of the disease but no convincing findings of remission. Good clinical results have been achieved in about 40% of the patients receiving the iv preparation and in about 35% of the patients receiving the oral preparation. A good clinical effect is considered to be the disappearance of uremia, the regression of metastases, the disappearance of metastatic pain, and the removal of the indwelling catheter.

Tables 2 and 3 show that most of the patients belong to stage IV according to the Veterans' Administration Cooperative Urological Research Group classification, and most of the carcinomas have a moderate to low degree of differentiation.

Table 4 shows the effect of estramustine phosphate on the serum acid phosphatase activity compared to that of the previous treatment. Thus, in a certain number of patients an

TABLE 1.—Effect of treatment with estramustine phosphate in patients resistant to previous therapy

Treatment route	Total No. of patients	Effect of treatment (No. of patients)		
		Good	Uncertain	None
Iv*	52	24	6	22
Oral†	38‡	12	7	16
Total	90	36	13	38

*Used from 1969 to Mar 1974.
†Used from 1971 to Mar 1974.
‡ Three patients are not included.

TABLE 2.—Grouping of patients treated with estramustine phosphate according to disease stage

Stage	Total No. of patients	No. of patients treated—	
		Iv*	Orally†
III	14	6	8
IV	118	70	48
Total	132	76	56

*Used from 1969 to Mar 1974.
†Used from 1971 to Mar 1974.

TABLE 3.—Degree of differentiation and distribution in patients treated with estramustine phosphate

Degree of differentiation	Total No. of patients	No. of patients treated—	
		Iv*	Orally†
Well	21	11	10
Moderate	53	25	28
Low	37	23	14
Total	111‡	59	52

*Used from 1969 to Mar 1974.
†Used from 1971 to Mar 1974.
‡Twenty-one patients are not adequately graded (17 treated iv and 4 treated orally).

additional depressant effect on serum acid phosphatase activity is noted. This implies an effect above that of the previous therapy. In most patients where the serum acid phosphatase activity decreases, a corresponding good clinical effect occurs.

Regression of metastases has been registered in a remarkably large number of patients (table 5). In 17 of the patients refractory to all earlier therapy, estramustine phosphate treatment was followed by regression of metastases in bones, lungs, and soft tissue. Regression of metastases is defined as a reduction in the diameter of the lesion by 50% of its original size. We have often been able to confirm the regression histologically or cytologically.

Only a few general side effects were registered indicating a generalized cytostatic effect of the compound.

In conclusion, both the iv and oral preparations have proved to give further palliation to patients in whom the tumor did not respond to previous therapy. The clinical effect is reported to be good in about 40% for the iv preparation and in about 30% for the oral preparation. Regression of metastases has been registered in about 25% of all patients. Because it appears that the iv compound might

TABLE 4.—Serum acid phosphatase activity in patients resistant to previous therapy*

Treatment route	Total No. of patients	No. of patients with acid phosphatase level—			
		Increasing	Decreasing	Continuously elevated	Normal
Iv	41	9	15	5	12
Oral	34	6	9	5	14

*No followup in 15 patients.

TABLE 5.—Regression of metastases in patients treated with estramustine phosphate

Regression of—	Patients resistant to conventional therapy (90 patients)		Patients receiving estramustine phosphate as primary treatment (28 patients)	
	Drug given iv (52 patients)	Drug given orally (38 patients)	Drug given iv (18 patients)	Drug given orally (10 patients)
Bone metastases	3	1	3	3
Pulmonary metastases	3	2	1	2
Soft tissue metastases	6	2	6	2
Total	12	5	10	7

TABLE 6.—Effect of primary treatment with estramustine phosphate

Treatment route	Total No. of patients	Effect of treatment (No. of patients)		
		Good	Uncertain	None
Iv*	24	24	0	0
Oral†	18	16	2	0
Total	42	40	2	

*Used from 1969 to Mar 1974.
†Used from 1971 to Mar 1974.

be somewhat more effective, we currently start the therapy using the iv route and afterwards give it orally.

Table 6 presents the preliminary results of a clinical trial using estramustine phosphate as initial therapy in patients with far-advanced rapidly growing prostatic carcinoma (stage IV). So far, the clinical evaluation is very promising. Every patient is treated for at least 6 months. In this series we have obtained good clinical results in about 90% of the patients.

Design of Spindle Poisons Activated Specifically by Prostatic Acid Phosphatase (PAP) and New Methods for PAP Cytochemistry [1,2]

Arnold M. Seligman,[3,4] Nancy J. Sternberger,[4] Buddha D. Paul,[4] Alan E. Friedman,[4] W. Allen Shannon, Jr.,[4] Hannah L. Wasserkrug,[4] Robert E. Plapinger,[4,5] and Dwo Lynm [4,6,7,8]

SUMMARY

By taking advantage of the structural requirements of the substrates for prostatic acid phosphatase (PAP), which consist of steric hindrance and the presence of basic nitrogen in the molecule, potential cytotoxic agents (spindle poisons) are being designed that will become enzyme activated specifically by PAP. Colchicine has been converted to colchiceinamides of substituted ethanolamines and *o*-phosphoethanolamines. The rate of hydrolysis of the latter by human prostatic tissue as compared to the rate of hydrolysis by human kidneys (P/K ratio) is given and indicates a significant degree of specificity for PAP. Some preliminary toxicity data in mice are also given. New thiocolchicine derivatives with phosphates on ring B are also being prepared for study and some preliminary toxicity data are given. The observation in biochemical experiments that phosphorylcholine is a very specific substrate for PAP has led us to develop specific cytochemical methods for PAP for both light and electron microscopy. Preliminary observations are given and good evidence is provided that PAP is not a lysosomal enzyme, unlike other acid phosphatases. Furthermore, PAP is to other acid phosphatases what the cholinesterases are to other esterases. Since the acid phosphatase that is able to hydrolyze phosphorylcholine is characteristic of prostatic epithelium, this is the acid phosphatase that is referred to by the designation of PAP. Other acid phosphatases (both lysosomal and nonlysosomal) in prostatic epithelial cells are not demonstrated by this substrate and hence are not included in this designation.

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Our research program is concerned with the design and synthesis of new chemotherapeutic agents for prostatic carcinoma. The high ac-

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[3] American Cancer Society Professor of Research Oncology and Cell Biology.

[4] Department of Research Oncology and Cell Biology, Sinai Hospital of Baltimore, Inc., and the Department of Surgery, The Johns Hopkins University School of Medicine, Baltimore, Md.

[5] Responsible for work on alkylating agents. *Present address*: US Customs Laboratory, Baltimore, Md.

[6] Responsible for work on stable nitrogen free radicals. *Present address*: Swiss Federal Institute of Technology, Zürich, Switzerland.

[7] The authors would like to acknowledge Edward S. Parelhoff and Mrs. Dale S. Jeffers for their technical assistance and Mrs. Sandi A. Kellman for the preparation of the manuscript. LD50s for the stable nitrogen free radicals and the phosphates of their leuco forms were determined in mice by Dr. Yale H. Caplan. Human and baboon prostate and kidney were obtained through the courtesy of the Medical Examiner of Maryland and Dr. Gerald P. Murphy, Roswell Park Memorial Institute, Buffalo, NY, respectively.

[8] *Reprint requests to*: Arnold M. Seligman, MD, Sinai Hospital of Baltimore, Inc., Belvedere Ave at Greenspring, Baltimore, Md 21215.

tivity of prostatic acid phosphatase (PAP) in human prostatic epithelial cells and in human prostatic adenocarcinoma has provided us with the possibility of designing agents that could be enzyme activated to cytotoxicity. Since the structural requirements of substrates for PAP differ from those of other acid phosphatases in man, the possibility exists of designing toxic agents that will have a high degree of specificity in vivo for prostatic epithelial cells and prostatic adenocarcinoma cells as compared to that for vital cells of the body.

A byproduct of this work is the finding that phosphorylcholine is a very specific substrate for PAP. This has led to the development of specific cytochemical methods for both light and electron microscopy.

METHOD

It was important at first to develop an in vitro test for comparing the specificity of various substrates for PAP. For this purpose the rate of hydrolysis of *o*-phosphates of various substrates by human prostatic tissue as compared to that by human kidney cortex (P/K ratio) was determined at pH 4.8 with homogenized prostate. Acid and alkaline phosphatases were determined with homogenized kidney at pH 4.8 and 9.0 respectively. The ratio was determined on a per milligram wet weight basis of each. Homogenates were prepared at a concentration of 200 mg of tissue/ml of 0.05 M tris-buffer at pH 7.0. Homogenates were centrifuged at 8000 rpm for 15 minutes and were stored in aliquots at –15°C for up to 6 months without changes in activity. The prostatic homogenate was diluted tenfold to 100-fold with 0.1 M acetate buffer (pH 4.8). The kidney homogenate was used directly. Each homogenate (0.01–0.10 ml) was added to 1.0 ml of 0.1 M acetate buffer at pH 4.8 for acid phosphatase determinations or to 1.0 ml of 0.05 M tris-buffer at pH 9.0 for alkaline phosphatase determinations. The substrates (3 mg) were introduced in 0.1 ml of water. Three concentrations of each homogenate were incubated with excess substrate and the amount of substrate hydrolyzed was plotted versus the amount of homogenate. From the resulting linear relationship the reaction rate was calculated per milliliter of homogenate and converted to a per milligram wet weight basis. The reaction mixtures were incubated at 37°C for periods varying from 30 minutes to 24 hours, depending upon the substrates under study. Those that were not hydrolyzed within 4 hours were incubated for 24 hours in order to determine whether any hydrolysis was possible. Hydrolysis was measured colorimetrically by determination of phosphate (1) or, when possible, colorimetrically with chromogenic substrates. When phenols were produced color was developed from 2,6-dibromoquinone-4-chloroimide, and for naphthols azo dyes were obtained with the stable diazonium salt, diazo fast blue BBN. A Klett colorimeter was used.

RESULTS

It became clear that two structural features favored hydrolysis by PAP as compared to acid and alkaline phosphatase of kidney cortex. These features were the presence of basic nitrogen, especially a quaternary salt, and a certain degree of steric hindrance near the *o*-phosphate group. The latter could be provided by an *o*-isopropyl group or two *o*-methoxyl groups (table 1). However, a tertiary *o*-butyl group offered too much steric hindrance even for PAP. One of the very specific substrates for PAP is phosphorylcholine (P/K = 1000 and P/K_{Alk} = 200 after 1.5 hours of incubation). The natural substrate for this enzyme may be phosphorylcholine which Lundquist (2) has shown is present in semen in large amounts. The P/K ratios for a few of the substrates we have studied are given in table 1. The roles of nitrogen and steric factors are revealed. The striking difference in P/K ratios for other species is shown in table 2. The low values in rodents are due to the low content of PAP in their prostates and not to the high phosphatase content of their kidney cortices as shown by comparing tables 2 and 3.

Histochemistry

The observation that phosphorylcholine was a very specific substrate for PAP prompted us to develop a cytochemical method for this enzyme, using phosphorylcholine as substrate and lead ion as capture agent for liberated

TABLE 1.—Results obtained with homogenates of human prostate and kidney cortex with a variety of phosphate substrates*

Substrate	P/K ratio	P/K_{AIR} ratio
$(CH_3)_3N^+CH_2CH_2OPO_3Ca \cdot Cl^-$	1000	200
$(HOCH_2CH_2)_2NCH_2CONHCH_2CH_2OPO_3H_2$	400	160
$NH_2CH_2CH_2OPO_3H_2$	255	75
$NH_2C(CH_3)_2CH_2OPO_3H_2$	420	70
$NH_2CH_2CH(CH_3)OPO_3H_2$	460	75
$NH_2CH_2CH_2CH_2OPO_3H_2$	545	130
$NH_2CH(CH_3)CH(CH_3)OPO_3H_2$	630	120
$NH_2CH(COOH)CH(CH_3)OPO_3H_2$	555	200
$NH_2CH_2CH(C_6H_5)OPO_3Na_2$	330	5
$CH_3NHCH(CH_3)CH(C_6H_5)OPO_3H_2$	865	220
$CH_3NHCH_2(CHOPO_3H_2)_4CH_2OPO_3H_2$	155	220
$CH_2OHCH(OPO_3H_2)CH_2OH$ (β-glycerophosphate)	250	820
$CH_2=C(OPO_3Na_2)COOH$	30	110
2,6-Dimethoxyphenyl phosphate	330	600
2,6-Dibromophenyl phosphate	230	1300
2,6-Dimethylphenyl phosphate	200	200
2-Isopropylphenyl phosphate	310	580
Phenyl phosphate	130	455
1-Naphthyl phosphate	300	1750
2-Naphthyl phosphate	160	1100
2-Methyl-1-naphthyl phosphate	340	1100
(2-Phosphorylbenzyl) trimethyl ammonium ion	660	1100
Indoxyl phosphate	110	320
Naphthol AS phosphate	55	290

*Rates of hydrolysis on a per milligram wet weight basis were obtained at pH 4.8 for acid phosphatase and at pH 9.0 for alkaline phosphatase of kidney. The results are given as the ratio of prostate to kidney (P/K). The higher the P/K ratio the greater the specificity for PAP.

TABLE 2.—Comparison of P/K ratios for rodent, dog, monkey, baboon, and man with phosphorylcholine

Species	P/K ratio	P/K_{AIR} ratio	Length of incubation (hrs)
Man	1000	200	1.5
Baboon	1200	29	1.5
Stumptail monkey	690	40	1.5
Dog	130	1.0	2
Rat	2	0.5	3
Mouse	0.3	0.002	3

TABLE 3.—Estimated ratio of PAP activity per milligram of prostate as compared to that of the rat after 3 hours of incubation with phosphorylcholine

Species	Ratio
Mouse	0.2
Rat	1
Dog	60
Stumptail monkey	680
Baboon	1100
Man	1200

phosphate ion as used by Gomori (3) for other acid phosphatases with β-glycerophosphate. Lead phosphate was converted to black lead sulfide for light microscopy. The details of the methods and the results for both light and electron microscopy will be published elsewhere. It is of interest to mention here that at pH 5.0, comparing glutaraldehyde-fixed rat prostate, kidney, and liver, with substrates phosphorylcholine and β-glycerophosphate, all acid phosphatases were demonstrated in 2 hours with β-glycerophosphate (fig 1[*b, d, f*]). However, acid phosphatase activity with phosphorylcholine was demonstrated only in prostatic epithelium (fig 1[*a, c, e*]). This was also the case with dog and primate prostatic epithelium, where a high content of PAP necessitated the reduction of the incubation time to 30 and 5 minutes respectively in order to avoid very dense overstaining.

The demonstration of some phosphatase activity at pH 5.0 with phosphorylcholine in brush border of rat kidney tubules (fig 1*c*) only after prolonged incubation (2 hours) is indicative of alkaline phosphatase activity. Although the pH is not optimal for alkaline phosphatase, the enzyme concentration in brush border is very high. It is interesting that at pH 5.0 rat alkaline phosphatase is able to hydrolyze phosphorylcholine but not β-glycerophosphate (*see* table 1).

Especially noteworthy was the demonstration with β-glycerophosphate of large droplets or lysosomes which were numerous in the kidney (fig 1*d*) and liver (fig 1*f*) and sparse in the prostate (fig 1*b*). No staining of these lysosomal structures was seen in any of these organs with phosphorylcholine after 2 hours of incubation. The liver was completely unstained (fig 1*e*) whereas the kidney showed ghosts of brush border staining (fig 1*c*) indicative of alkaline phosphatase activity although at an unoptimal pH. Brush border was not stained by β-glycerophosphate at the same pH. In the prostate, small secretory granules, as well as the alveolar contents, were stained (fig 1*a*). These experiments confirm the specificity of phosphorylcholine for PAP and provide us with new cytochemical methodology for specifically studying the precise localization of this enzyme. Preliminary observations in electron microscopy using this new method confirmed that PAP is not a lysosomal enzyme, unlike other acid phosphatases. Results with alkaline phosphatase at pH 9.0 are shown in figure 2 (*a–d*) and indicate that the two substrates behave similarly at alkaline pH.

It appears that the relation of PAP to other acid phosphatases is similar in the structural requirements for specificity of quaternized nitrogen in its substrates as those of cholinesterases are to other esterases. Ultrastructural study of prostatic carcinoma with a method specific for PAP should be of interest. Results obtained so far justify reference to PAP as including only the acid phosphatase which is able to hydrolyze phosphorylcholine at pH 5.0 and is therefore characteristic of the prostatic epithelium.

Alkylating Agents

Our first efforts were directed at the design of alkylating agents which would have a degree of enzyme activation by PAP. However, in vitro studies indicated a high degree of inactivation of the phosphatases, including alkaline phosphatase, by the alkylating agents. We observed that estramustine phosphate [9] (4) is also able to inactivate all the phosphatases in human prostate and kidney cortex at pH 4.8 and 9.0. Only after inactivation of the alkylating ability of estramustine phosphate by heating an aqueous solution at pH 9.0 and 100°C for 3 hours was it possible to demonstrate that prostatic tissue and kidney cortex could

[9] Estramustine phosphate: NSC-89199; CAS reg. No. 4891-15-0; estra-1,3,5(10)-triene-3,17β-diol, 3-[bis(2-chloroethyl)carbamate] 17-(dihydrogen phosphate); Estracyt.

hydrolyze the 17-phosphoryl group at acid and alkaline pH. The P/K ratios were 130 and 660 respectively. It is apparent that the 17-phosphoryl group of estramustine phosphate plays no other role than that of providing water solubility.

Stable Nitrogen Free Radicals

Our next effort was to determine if the cytotoxic compounds with stable nitrogen free radicals (5) could be converted to nontoxic phosphates after reduction to the leuco form, and whether the phosphates would be specifically hydrolyzed by PAP. For this purpose 2,2,-6,6-tetramethylpiperidine-1-oxyl (5) and di tert butyl nitroxide (3) were reduced to their leuco forms and phosphorylated. Their P/K ratios were 40 and 100 respectively at pH 4.8 and 55 and 100 respectively at pH 9.0. The poor hydrolysis of these phosphates was due to too much steric hindrance for PAP as well as for the other phosphatases in man. These ratios are not high enough to give promise of significant specificity for prostatic carcinoma. This rendered less impressive the finding in mice that the ratios of the LD50 of leuco phosphates to the LD50 of free radicals were 3.3 and over 14 respectively.

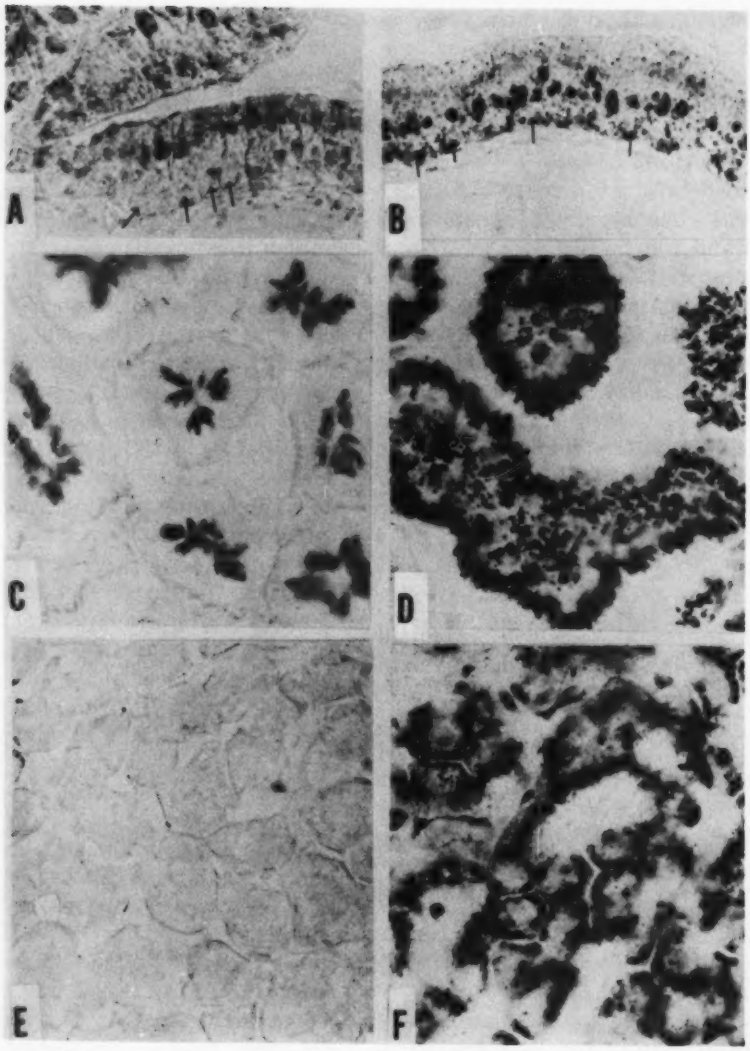
Spindle Poisons

Our present approach deals with attempts to convert the spindle poisons, podophyllotoxin (NSC-24818) (I) (6) and colchicine (NSC-757; CAS reg. No. 7006-15-7) (III) (fig 3), to less toxic agents which will be specifically activated by PAP. These antimitotic substances halt mitosis by interfering with the function of microtubules by interaction with microtubule protein (tubulin) (7).

Podophyllotoxin (I) has a benzylic hydroxyl group capable of being phosphorylated (II). We have done this, and it was found that II was not hydrolyzed by PAP and was only minimally hydrolyzed by the phosphatases of kidney cortex. Also disappointing was the finding in mice that the maximum tolerated dose (MTD) of II is only 75 mg/kg as compared to 50 mg/kg for I, an MTD ratio of only 1.5. Other analogs are being designed and prepared that may provide both significantly higher P/K ratios and higher MTD ratios (MTD of the phosphorylated derivative to the MTD of the unphosphorylated derivative). The preparation of the benzylic chloro derivative of I, with an MTD of 50 mg/kg, should provide us with a useful starting material for some new phosphate analogs.

Since colchicine (III) (fig 3) is a methylated tropolone, the methoxyl group of ring C reacts more like an ester than an ether and is readily replaced on heating with amines (8). This afforded the opportunity to prepare a variety of substituted ethanolamines that could be *o*-phosphorylated for determination of their P/K ratios (table 1). These determinations were made with human tissue. One of the very specific substrates (phosphorylcholine) for human PAP was compared in other species with the striking results shown in tables 2 and 3. That the low ratios, especially in rodents, were not due to the high phosphatase activity of kidney cortex but rather to the relatively low activity of prostatic epithelium, is shown in table 3. The data in tables 2 and 3 show that phosphatase activities in kidneys of various species are nearly alike in contrast to PAP activity in their prostatic epithelium. Some of the ethanolamine derivatives that look promising in this assay were reacted with colchicine to form the corresponding colchiceinamide derivatives for comparison of their toxicity with colchicine in mice (table 4). Also included are some new thiocolchicine derivatives. For this purpose the MTD was obtained by intraperitoneal (ip) injection in adult male white mice. Table 4 shows that most of the ethanolamine analogs of ring C were 10% or less as toxic as colchicine.

Some of the promising substituted *o*-phosphoryl ethanolamines were reacted with colchicine by refluxing an aqueous solution of equimolar quantities at pH 9.0 for 1–4 days. The pH was obtained by adding sodium bicarbonate until the pH was 7.2–7.5 with glass electrode. When CO_2 was lost on heating, the pH rose to 9.0. Thin-layer chromatography was used to determine when the reaction was complete. The reaction mixture was stripped to dryness in vacuo, and the product was extracted with chloroform, filtered, concentrated,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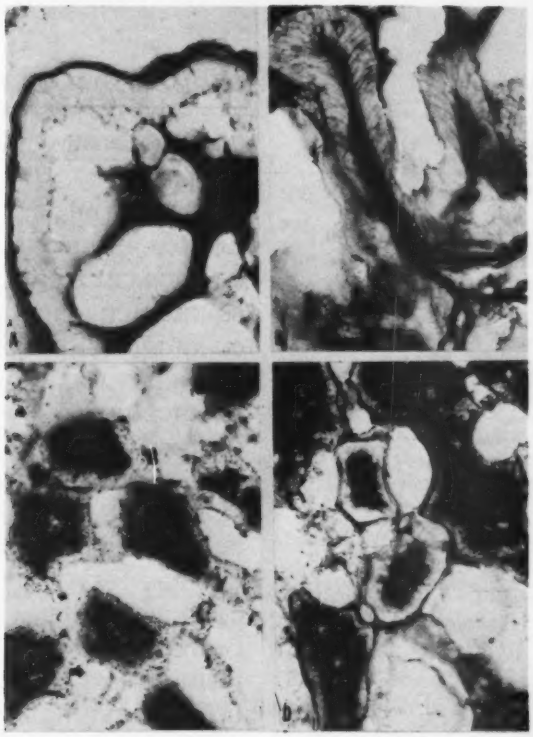

FIGURE 2.—Alkaline phosphatase in prostate and kidney of the rat at pH 9.0. Incubation, 2 hrs. Figures *A* and *C* are with phosphorylcholine as substrate and figures *B* and *D* are with β-glycerophosphate. (*A*) Some alkaline phosphatase activity is seen in the intercellular membranes of prostatic cells and heavily in the lamina propria and alveolar contents. Substrate: phosphorylcholine. (*B*) Somewhat heavier alkaline phosphatase activity is seen in prostatic cells with β-glycerophosphate as substrate than in cells with phosphorylcholine (compare with fig 2*A*). (*C*) Kidney proximal tubule brush border stains heavily for alkaline phosphatase activity with phosphorylcholine as substrate. Marked differences noted with acid phosphatase are not seen with alkaline phosphatase. (*D*) Somewhat heavier staining of kidney proximal tubular brush border for alkaline phosphatase activity is seen with β-glycerophosphate as substrate. Marked differences noted with acid phosphatase are not seen with alkaline phosphatase.

←

FIGURE 1.—Acid phosphatases in prostate, kidney, and liver of the rat at pH 5.0 using the lead capture reaction for phosphate of Gomori (3). Incubation, 2 hrs. Figures *A*, *C*, and *E* are with phosphorylcholine as substrate and figures *B*, *D*, and *F* are with β-glycerophosphate. Slight artifactually stained cell nuclei due to some affinity to nucleic acid for lead ion will be seen in some of the photomicrographs. This is an acknowledged weakness of the Gomori technique. (*A*) PAP activity is seen in secretory granules and dust-like patches distributed mainly in the apex of the prostatic epithelial cells. No stained large lysosomal droplets are seen (compare with fig 1*B*). A few lightly stained cell nuclei (artifacts) can be seen near the base of the columnar cells (arrows). Substrate: phosphorylcholine. (*B*) PAP in secretory granules is seen in the apical region of prostatic epithelial cells. In addition, there are densely stained large lysosomal droplets in supranuclear regions of the cell as well as perinuclear granules. Some artifactually lightly stained cell nuclei can be seen (arrows). Substrate: β-glycerophosphate. (*C*) Kidney tubules show no PAP or lysosomal staining. The brush border which has a very high alkaline phosphatase activity is able to slowly hydrolyze phosphorylcholine (2 hrs) at unoptimal pH 5.0. This is not the case with β-glycerophosphate (compare with fig 1*D*). Substrate: phosphorylcholine. (*D*) Kidney tubules show abundant acid phosphatases in granules and lysosomes (large droplets). Faintly stained cell nuclei (artifacts) can be seen. No brush border stain is present (compare with fig 1*C*). Substrate: β-glycerophosphate. (*E*) Liver cells show no PAP or other acid phosphatase activity (compare with fig 1*F*). Substrate: phosphorylcholine. (*F*) Liver cells show abundant acid phosphatases in granules and in lysosomes (large droplets) which are especially abundant adjacent to the bile canaliculi seen between cells (compare with fig 1*E*). Substrate: β-glycerophosphate.

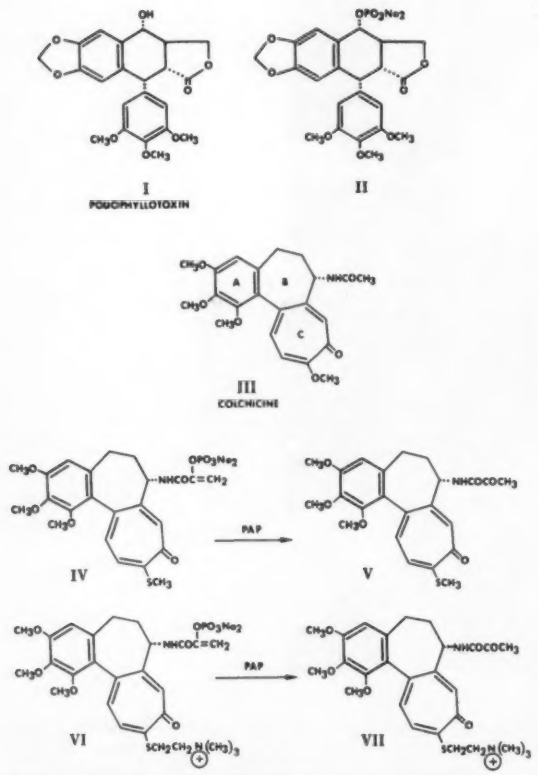

FIGURE 3.—Chemical structures of potential spindle poisons and their conversion to potentially less toxic agents to allow specific activation by PAP.

and precipitated with ether. Some of these new compounds are shown in table 5. It was noted that the P/K ratios of the colchicine analogs are somewhat higher than the corresponding phosphates given in table 1, probably due to increased steric hindrance produced by the bulky colchicine moiety.

Equally as important as the P/K ratio for predicting possible clinical usefulness is the ratio of MTD of the phosphorylated derivative to the MTD of the unphosphorylated derivative. As will be seen in table 5, this ratio in mice was unimpressive with all of the phosphorylated derivatives. The polyphosphates were prepared in the hope that the small reduction in toxicity noted with the monophosphates would be multiplied by the number of phosphate groups. As may be seen in table 5, this was not accomplished with the polyphosphorylated glucamine derivative.

TABLE 4.—MTD of cholchicine derivatives in male mice

Colchicine derivatives	MTD (mg/kg)
Colchicine	3.0
Col-$NHCH_2CH_2OH$	75
Col-$N(CH_3)CH_2CH_2OH$	75
Col-$NHCH_2CH_2CH_2OH$	300
Col-$NHCH(CH_3)CH(CH_3)OH$	100
Col-$NHCH(COOH)CH(CH_3)OH$	75
Col-$N(CH_2CH_2OH)_2$	100
Col-$NHCHOHCH_2OH$	300
Col-$NHCH_2CH(C_6H_5)OH$	75
Col-$N(CH_3)CH(CH_3)CH(C_6H_5)OH$ (levo)	25
Col-$N(CH_3)CH_2(CHOH)_4CH_2OH$	120
Col-$NHCH(CH_3)CH(OH)C_6H_3(OH)_2$	90
Col-NH_2	60
Col-$NHCH_2CH_2N(CH_3)_2$	240
Col-$NHN(CH_3)_2$	15
Deacetyl Col-$NHN(CH_3)_2$	90
Deacetyl Col-$NHN(CH_3)_2$ glycolamide	30
Deacetyl Col-$NHN(CH_3)_2$ pyruvamide	90
Deacetyl Col-$NHN(CH_3)_2$ acetoacetamide	90
Col-$NHCOCH_2Cl$	90
Thiocolchicine	3.0
Col-$SCH_2CHOHCH_2OH$	270
Deacetyl thiocolchicine	150
Deacetyl thiocolchicine chloracetamide	15
Deacetyl thiocolchicine pyruvamide (V)	7.5
Deacetyl thiocolchicine lactamide (levo)	15
Deacetyl thiocolchicine glycolamide	9
Deacetyl thiocolchicine acetoacetamide	15
Col-$SCH_2CH_2N^+(CH_3)_3I^-$	30
Col-$NHCH_2CH_2N^+(CH_3)_3I^-$	210

Although MTD ratios could be expected to be higher in man, where P/K ratios are very much higher than in the mouse (tables 2 and 3), we are trying to design compounds with higher MTD ratios in mice before proceeding to higher animals.

Modifications of ring B are also being made in an attempt to improve the MTD ratio. We began by stabilizing ring C to hydrolysis by conversion of colchicine to the unsymmetrical dimethylhydrazide and to thiocolchicine (9)

TABLE 5.—Colchiceinamide derivatives containing phosphoryl groups*

Colchiceinamide phosphates	P/K ratio	P/K_{Alk} ratio	MTD (mg/kg)	$\frac{\text{MTD } PO_4}{\text{MTD OH}}$
C-$NHCH_2CH_2OPO_3Na_2$	500	170	150	2.0
C-$NHCH_2CH_2CH_2OPO_3Na_2$	770	1770	120	0.4
C-$NHCH(CH_3)CH(CH_3)OPO_3Na_2$	780	1010	150	1.0
C-$NHCH(COOH)CH(CH_3)OPO_3Na_2$	175	70	75	1.0
C-$NHCH_2CH(C_6H_5)OPO_3Na_2$	480	910	75	1.0
C-$N(CH_3)CH(CH_3)CH(C_6H_5)OPO_3Na_2$	980	2400	90	4.0
C-$N(CH_3)CH_2(CHOPO_3Na_2)_5H$	250	810	200	2.0

*Rate of hydrolysis by human PAP as compared to that of acid and alkaline phosphatase of kidney cortex (P/K) is tabulated as well as the MTD in male mice and the ratio of MTD of phosphorylated derivative to MTD of unphosphorylated derivative $\left(\frac{\text{MTD } PO_4}{\text{MTD OH}}\right)$.

which were then deacetylated (10). The deacetylated products had MTDs of 90 and 150 mg/kg respectively (table 4). They were then acylated with pyruvic acid and acetoacetic ester to determine whether the toxicity was restored to that of the colchiceine dimethylhydrazide (MTD = 15 mg/kg) and thiocolchicine (MTD = 3 mg/kg). The MTDs obtained for the new analogs are shown in table 4. The results with deacetyl thiocolchicine pyruvamide (V) have encouraged us to attempt the preparation of the enol phosphate analog (IV) (fig 3). The rationale for this was that the enol phosphate might have low toxicity and after enzymatic hydrolysis would revert to the keto form which would have significant toxicity (MTD = 7.5 mg/kg). It was hoped that this keto compound would be much more toxic than the hydroxy form which had too little toxicity in the ring C analogs. Partial confirmation of this hypothesis was obtained after preparing deacetyl thiocolchicine glycolamide and lactamide. The MTD for both turned out to be only a little higher (MTD = 9–15 mg/kg), as compared to an MTD of 7.5 mg/kg for the pyruvamide (table 4).

The lactamide phosphate and the glycolamide phosphate of deacetyl thiocolchicine had MTDs of 150 and 75 mg/kg respectively. Although their MTD ratios were 10 and 8, their P/K ratios were 260 and 390 respectively. To test the ability of a basic group in ring C to raise the P/K ratio, we prepared the glycolamide and its phosphate of deacetylated colchiceine dimethylhydrazide. Although the MTD ratio was 7.5 (225/30), the P/K ratio of the phosphate was only 200. This suggested to us that nothing short of quaternized nitrogen in the side chain of either ring C or B would give us P/K ratios that were high enough. We expected to lose considerable toxicity thereby, as indicated in the MTD of the colchicine ring C thiocholine analog (table 4). Its MTD is ten times that of thiocolchicine. Deacetylation of ring B and acylation, as was done for the colchiceine dimethylhydrazide and for thiocolchicine, will produce the new agent (VII) and the enol phosphate (VI) (fig 3). These are in preparation because the anticipated high P/K ratios may be more important than the favorable MTD ratios expected with the thiocolchicine analogs (IV and V).

Another parameter that is being studied is the best way to determine the antimitotic effect expressed as a minimum effective dose (MED). Preservation of the antimitotic activity was shown with some of the colchiceinamides in preparations of mice testes, according to Bergner's procedure (11), and in Sarcoma 180 within 24 hours after ip injection. We are now examining the usefulness of 3-day cultured human lymphocytes. Using this technique, the MED of colchicine is about 10^{-7} M.

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Initial Chemotherapeutic Trials in Patients With Inoperable or Recurrent Cancer of the Prostate

Francisco Tejada and Martin H. Cohen [1,2,3]

SUMMARY

This paper describes a random study being conducted to compare the value of 5-fluorouracil plus diethylstilbestrol vs diethylstilbestrol alone and the value of 5-fluorouracil vs CCNU in the treatment of stage D adenocarcinoma of the prostate. We have established that similar normal values of acid phosphatase are encountered in serum and bone marrow of patients with nonmalignant and malignant diseases without osseous metastasis.

[Cancer Chemother Rep 59:243–249, 1975]

Since Huggins and Hodges' (1) initial observations, estrogen therapy or castration or both have been the mainstay of therapy for stage D adenocarcinoma of the prostate. Although symptomatic response to estrogen treatment is often dramatic, about 30% of the patients receiving this treatment are expected to die from their disease within 48 months after treatment (2). Despite the limitations of estrogen therapy in prostatic cancer, the use of chemotherapeutic agents has been studied only sporadically until the studies of the National Prostatic Cancer Project (NPCP) were started (3). In September 1973, the NCI-VA Medical Oncology Branch joined this effort and initiated a randomized chemotherapeutic study for advanced adenocarcinoma of the prostate, using a schedule and dosage of drugs that would permit comparison with other studies.

MATERIALS AND METHODS

For study purposes, patients with a diagnosis of stage D adenocarcinoma of the prostate were documented by biopsy or by two of the following tests: elevated serum or bone marrow acid phosphatase, positive bone survey, or positive technetium pyrophosphate bone scan. These patients were then divided into two groups for treatment (fig 1, Protocol MOB-73-02).

Group 1 patients, those who had not received diethylstilbestrol (DES)[4] or orchiectomy, were stratified by extent of disease, previous therapy, and level of serum acid phosphatase. They were randomly allocated to treatment with 1 mg/day of DES orally or with 1 mg/day of DES orally plus 600 mg/m^2 of 5-fluorouracil (5-FU)[5] intravenously (iv) every week × 4 weeks and 750 mg/m^2 iv every week thereafter if tolerated.

Group 2 patients, those who had received DES and/or orchiectomy, were stratified by extent of disease and level of serum acid phosphatase. They were randomly allocated to treatment with 5-FU as described for group

[1] National Cancer Institute-Veterans' Administration (NCI-VA) Medical Oncology Branch, Division of Cancer Treatment (DCT), National Cancer Institute (NCI), Washington, DC.

[2] Participating investigators in this study were John Bressette, Kenneth Sacks, Richard Simon, and the senior staff of the NCI-VA Medical Oncology Branch, DCT, NCI, Washington, DC.

[3] *Reprint requests to:* Francisco Tejada, MD, NCI-VA Medical Oncology Branch, VA Hospital, Washington, DC 20422.

[4] Diethylstilbestrol: NSC-3070; 4,4'-stilbenediol, α, α'-diethyl-; stilbestrol.

[5] 5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8. Supplied by Cancer Therapy Evaluation (CTE), DCT, NCI.

1 or to treatment with CCNU[6] (130 mg/m²) orally every 6 weeks with crossover treatment upon progression of disease.

Study parameters include performance status, changes in measurable masses of tumor (product of longest times widest perpendicular diameter), metastatic bone survey, bone scan, bone marrow aspiration and biopsy, and serum and bone marrow acid phosphatase (Colorimetric assay, Phosphatabs-Acid Quantitative Method, Warner-Chilcott, Morris Plains, NJ).

Response is measured with set criteria for objective response (complete response and partial response $\geq 50\%$ tumor regression in the absence of new lesions or progression elsewhere), subjective response (improvement in functional class by two categories), and progression.

RESULTS

From September 1973 to June 1974, 16 patients have been entered in this study and evaluable patients are shown in table 1. The numbers of evaluable patients are not adequate to evaluate the outcome of therapy at this time, but the toxicity so far encountered is of some interest and is summarized in table 2.

Since one of the study parameters was the level of serum and bone marrow acid phosphatase, we were interested in finding the range of values for this enzyme in bone marrow serum from patients who had prostatic cancer as compared with other diseases. In measuring acid phosphatase we used the Phosphatabs-Acid Quantitative assay with a normal value of 0.5–5.0 Babson-Read units (given by the manufacturer).

Our results in ten patients with nonmalignant diseases showed a mean serum acid phosphatase value of 1.1 ± 0.6 Babson-Read units. The corresponding bone marrow acid phosphatase values were 1.6 ± 1.1 Babson-Read units (table 3).

In 13 patients having different malignant diseases in whom metastasis to the bone or bone marrow was not found by bone marrow aspirate, biopsy, bone survey, or bone scan, the serum acid phosphatase value showed a mean of 0.9 ± 0.6 Babson-Read units and bone marrow values of 1.6 ± 1.2 Babson-Read units (table 4).

In 12 patients with malignant diseases whose primary tumor originated in the bone marrow or had metastasized to the bone or bone marrow, the serum acid phosphatase value was 1.6 ± 1.2 Babson-Read units, but the bone marrow acid phosphatase level showed wide variation from a low of 0.4 to a high of 12.3 Babson-Read units (table 5). The initial serum and bone marrow acid phosphatase values of our patients entered in Protocol MOB-73-02 are given in table 6.

[6] CCNU: NSC-79037; CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-. Supplied by CTE, DCT, NCI.

TABLE 1.—NCI-VA Medical Oncology Branch Protocol MOB-73-02

Treatment	No. of patients—			
	Entered in study	Not yet evaluable	Lost to study	Evaluable
DES	4	—	—	4
DES + 5-FU	3	—	1	2
5-FU	4	—	—	4
CCNU	4	1	1	2
Progress to—				
CCNU	—	—	—	—
5-FU	1	—	—	1
Totals	16*	1	2	13

*No. of patients entered from September 1973 to June 1974.

TABLE 2.—NCI-VA Medical Oncology Branch Protocol MOB-73-02

Treatment	Patient No.	Toxic effects of therapy*			
		Heart failure	Wbc count	Platelet count	Gastro-intestinal
DES	1	—	—	—	—
	2	2+	—	—	—
	3	2+	—	—	—
	4	—	1+ (radiation)	—	—
DES + 5-FU	5	1+	2+	2+	1+
	6	—	2+	—	—
5-FU	7	—	1+	—	—
	8	—	—	3+	1+
	9	—	2+	1+	—
	10†	—	4+	3+	2+
CCNU	11	—	3+	2+	—
	12	—	2+	1+	—
Progress to—					
CCNU	—	—	—	—	—
5-FU	12	—	—	—	—

*Toxicity	Wbc (cells/mm³)	Platelets (cells/mm³)	Degree
1+	<4000	<150,000	Slight
2+	<3000	<100,000	Moderate
3+	<2000	< 75,000	Marked
4+	<1000	< 50,000	Life threatening

†Developed pneumonia and sepsis and died.

TABLE 3.—Acid phosphatase levels in nonmalignant diseases*

Diagnosis	Acid phosphatase level in—†	
	Peripheral blood	Bone marrow
Iron deficiency anemia	1.2	0.5
Iron deficiency anemia	1.7	3.0
Anemia, chronic alcoholism	0.6	1.5
Anemia, diabetes	1.7	2.1
Anemia, tuberculosis	1.9	3.3
Tuberculosis	1.9	2.7
Diabetes, uremia	0.7	0.7
Lymphoma in remission	0.5	0.5
Calcified lung nodule	1.2	1.7
Ankylosis spondylitis	0.8	2.1
Mean	1.1	1.6
Standard deviation	0.6	1.1

*Bone marrow aspirate and biopsy negative for malignancy.
†Normal range = 0.5–5.0 Babson-Read units.

TABLE 4.—Acid phosphatase levels in malignant diseases*

Diagnosis	Acid phosphatase level in—†	
	Peripheral blood	Bone marrow
Seminoma, stage II	1.2	1.2
Lymphoma	0.5	0.5
Epidermoid carcinoma, lung	2.1	3.3
Epidermoid carcinoma, lung	0.2	0.8
Epidermoid carcinoma, lung	1.8	2.1
Epidermoid carcinoma, lung	1.0	0.7
Epidermoid carcinoma, lung	0.4	0.5
Epidermoid carcinoma, lung	1.0	2.7
Large-cell carcinoma, lung	0.6	3.0
Adenocarcinoma, lung	1.5	0.8
Adenocarcinoma, lung	1.2	4.0
Small-cell carcinoma, lung	1.2	1.5
Small-cell carcinoma, lung	0.8	1.3
Mean	0.9	1.6
Standard deviation	0.6	1.2

*Bone marrow aspirate, biopsy, bone survey, and bone scan all negative for bone metastasis.
†Normal range = 0.5–5.0 Babson-Read units.

TABLE 5.—Acid phosphatase levels in malignant diseases*

Diagnosis	Acid phosphatase level in—†	
	Peripheral blood	Bone marrow
Multiple myeloma	0.5	0.5
Acute myelocytic leukemia	0.3	0.4
Bladder cancer	1.5	3.3
Large-cell carcinoma, lung	0.6	0.8
Adenocarcinoma, lung	1.7	1.9
Adenocarcinoma, lung	3.0	2.7
Adenocarcinoma, lung	3.7	1.7
Adenocarcinoma, lung	1.2	0.7
Adenocarcinoma, lung	2.1	4.7
Small-cell carcinoma, lung	1.0	12.3
Small-cell carcinoma, lung	1.0	6.2
Histiocytic lymphoma	3.7	6.6
Mean	1.6	3.2
Standard deviation	1.2	3.5

*Bone marrow aspirate, biopsy, bone survey, or bone scan was positive for bone metastasis.
†Normal range = 0.5–5.0 Babson-Read units.

TABLE 6.—Acid phosphatase and bone studies in stage D cancer of the prostate

Previous therapy	Acid phosphatase level in—*		Bone—†		Bone marrow—	
	Peripheral blood	Bone marrow	Survey	Scan	Aspirate	Biopsy
None	27.0	28.6	+	+	+	+
DES	42.0	124.0	+	+	+	+
CCNU	12.3	52.0	+	+	+	+
DES	40.0	174.0	+	+	+	+
DES, cyclophosphamide, methotrexate	210.0	250.0	+	+	+	+
Prostatectomy	13.3	45.0	+	+	+	+
DES	8.6	24.0	+	+	+	+
Prostatectomy	42.0	74.0	+	+	-	+
None	12.3	15.6	+	+	-	-
None	39.0	Dry	+	+	Dry	+
CCNU	5.4	11.8	+	+	-	-
DES	2.1	5.8	+	+	-	-
DES	4.3	Dry	+	+	Dry	-
Mean	32.7	67.0				
Standard deviation	53.4	77.5				

*Normal range = 0.5–5.0 Babson-Read units.
†Bone scan performed with technetium pyrophosphate.

DISCUSSION

Randomized trials used to test the effectiveness of chemotherapeutic agents in prostatic cancer are currently being conducted. The NPCP cooperative study (3) and other institutional studies sponsored by the NPCP are using as one arm of their studies the same dose and schedule of 5-FU that we used in our Protocol MOB-73-02, making future results easy to compare.

Since 5-FU has been one of the most widely used drugs which has some reported activity in the treatment of prostatic cancer (4),[7] we were interested in evaluating the effect of this drug when combined with DES in the treatment of stage D adenocarcinoma of the prostate (group 1, Protocol MOB-73-02).

We were equally interested in comparing 5-FU with a chemotherapeutic agent (like CCNU) that has not been used before in prostatic cancer in patients that have failed to respond to DES and/or orchiectomy (group 2, Protocol MOB-73-02). This study is currently being conducted and results will be forthcoming.

Thus far we note that treatment with DES in three of six patients in this protocol study was associated with a moderate degree of congestive heart failure which was easily controlled with a dietary regimen and diuretics. In general, the use of 5-FU and CCNU has produced moderate degrees of gastrointestinal and hematologic (white blood cell [wbc] and platelet) toxic effects (table 2). However, patient No. 10 received four courses of weekly 5-FU at 600 mg/m² and then four courses of weekly 5-FU at 750 mg/m² without having toxic reac-

[7] Slavik M, and Carter SK. Single agents in prostatic cancer—a quick review. Prepared for the NPCP (Murphy GP, Chairman) by the CTE, DCT, NCI, Bethesda, Md.

tions or achieving any response. Therefore, the patient was given two weekly doses of 5-FU at 1000 mg/m² as indicated in the initial design of Protocol MOB-73-02. This patient had thrombocytopenia of 55,000 cells/mm³ and leukopenia of 700 cells/mm³ with subsequent development of pneumonia and sepsis that did not respond to vigorous antibiotic therapy and resulted in death. Therefore, we have modified the protocol (fig 1), limiting the maximal weekly dose of 5-FU to 750 mg/m², and no further life-threatening toxicity has been encountered.

Finally, we are exploring the sensitivity of bone marrow acid phosphatase for the evaluation of response to treatment based on its established diagnostic value for metastatic disease (5–7). From our studies of bone marrow acid phosphatase in patients with nonmalignant and malignant diseases (tables 3 and 4), we concluded that normal values for acid phosphatase in bone marrow are 0.5–5.0 Babson-Read units using the Phosphatabs-Acid Quantitative assay. Some patients with osseous metastasis and patients with prostatic cancer and bone metastasis (tables 5 and 6) have higher marrow values, suggesting in the latter group that bone marrow acid phosphatase would be more sensitive for evaluating response and progression than serum acid phosphatase as illustrated by patients No. 2 (fig 2) and No. 12 (fig 3) who are currently in our study.

Patient No. 2 received treatment with DES

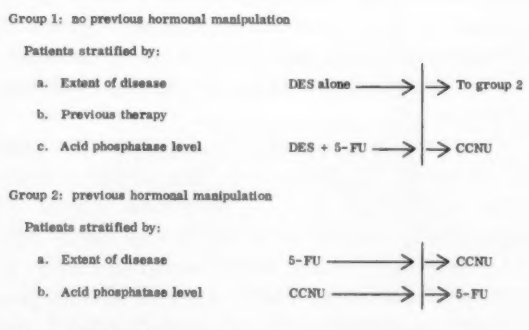

FIGURE 1.—Initial chemotherapeutic trials in patients with inoperable or recurrent cancer of the prostate (Protocol MOB-73-02, NCI-VA Medical Oncology Branch, Washington, DC, Sept 1973).

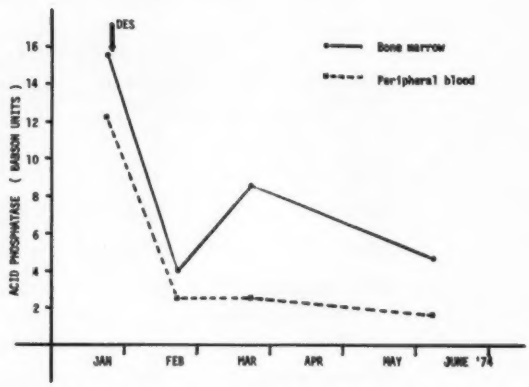

FIGURE 2.—Acid phosphatase levels in patient No. 2.

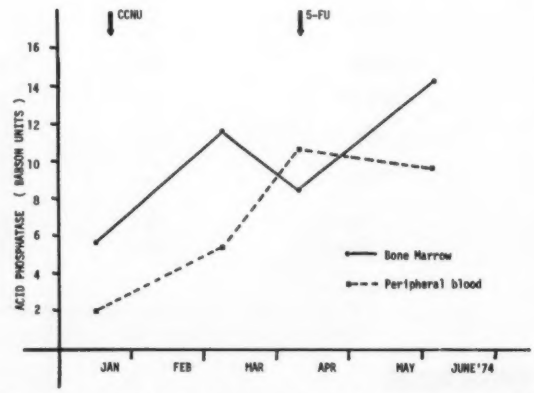

FIGURE 3.—Acid phosphatase levels in patient No. 12.

and had abnormal values of both serum and bone marrow acid phosphatase. While receiving treatment, the serum acid phosphatase value became normal within 1 month of therapy. The bone marrow acid phosphatase value decreased but has not yet reached "normal" levels. These changes correspond to a marked clinical improvement.

Patient No. 12 started treatment after new lesions were documented by a bone scan, and he had a normal serum acid phosphatase level while the bone marrow acid phosphatase level was 5.8 Babson-Read units. The patient has continuously shown clinical progression of disease and the levels of bone marrow acid phosphatase have generally been higher than the corresponding serum acid phosphatase levels.

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General Discussion: Treatment of Prostatic Cancer[1,2]

M. Tannenbaum: Table 1 refers primarily to the problem of prolactin in human prostatic tissue. There are several people at our institution who are using L-dopa and various other medications for the depression of prolactin secretion. Dr. Andrew Frantz has published a definitive article on prolactin secretion (1). We have fluoresceinated ovine prolactin antiserum and have studied normal nonmalignant prostatic tissue as well as several carcinomas of the prostate. You can see that if one uses immunofluorescent techniques similar to those I described yesterday (*see* General Discussion: Etiology and Prevention of Prostatic Cancer, p 73), none of the 30 benign prostatic hypertrophy configurations out of 37 cases had any staining with fluoresceinated anti-serum to ovine prolactin. In addition, there was no staining in the seven cancer cases. We have also tested various types of controls. Perhaps the prolactin binding receptor protein in these cells is oversaturated. We have also done elution studies which were negative. Perhaps there are no ovine prolactin receptor proteins within either nonmalignant or malignant prostatic epithelium. Fluoresceinated ovine prolactin was added and we did not get any binding by the prostatic tissue. One might question whether these are valid controls. In studies of human breast tissue we did get binding with our ovine prolactin antiserum in very specific places within both normal and malignant breast epithelium. The staining was usually in the so-called basaloid cells, which are not present in most prostatic cancers but are present in benign prostatic hypertrophy. We have also stained the prolactin cells in the human pituitary gland. It might therefore be concluded that although prolactin may be important in animals, it is, at the present time and with the present methodology, not important as a factor in regulating human prostatic tissue. We have antiserum to the beta chain of human chorionic gonadotropin. The antigen which has been used to develop this antiserum was used for the amino acid sequencing of this molecule. This antigen has some biologic properties of follicle-stimulating hormone (FSH) and luteinizing hormone (LH). We get a tremendous amount of immunofluorescent staining in the basaloid cells of human chorionic gonadotropin and also in certain types of prostatic cancer. I think a more profitable area, if one wants to go into the various types of pituitary tropic factors that are important in the regulation of prostatic growth, might be studies on FSH and LH. We are now concurrently pursuing this with Charles Ely at our institution who has done a considerable amount of work on FSH and LH. We also have lysosome antiserum that we have obtained from Elliott Osserman. Interestingly enough, the cases of benign prostatic hypertrophy have been nega-

[1] The following investigators participated in this discussion: *M. Tannenbaum* (Columbia University, New York, NY), *W. Whitmore* (Memorial Hospital for Cancer and Allied Diseases, New York, NY), *D. Coffey* (The Johns Hopkins Hospital, Baltimore, Md), *W. Voigt* (University of Miami School of Medicine, Fla), *A. Mittelman* (Roswell Park Memorial Institute, Buffalo, NY), *J. Grayhack* (Northwestern University Medical School, Chicago, Ill), *M. Bagshaw* (Stanford University School of Medicine, Palo Alto, Calif), *C. Merrin* (Roswell Park Memorial Institute, Buffalo, NY), *R. Gibbons* (Virginia Mason Foundation for Medical Education and Research, Seattle, Wash), and *M. Pollard* (University of Notre Dame, Ind).

[2] *Reprint requests to:* Mrs. Pamela W. Hutchins, *Cancer Chemotherapy Reports*, National Cancer Institute, Blair Bldg, Rm 3A05, Silver Spring, Md 20910.

TABLE 1.—Direct immunofluorescent study of human prostatic glands: benign and malignant*

Antibodies	No. of positive cases/No. examined	
	Benign	Malignant
Ovine prolactin	0/30†	0/5
Lysozyme	0/8	3/7
Herpesvirus type I	12/23	3/6
Herpesvirus type II	11/21	3/6
Cytomegalovirus	1/10	0/4
Human chorionic gonadotropin	2/3	—

*Four clinically normal prostates were not included because they were essentially immunologically negative and their number was too small.

tive, while several of the cases of prostatic cancer have been positive.

W. Whitmore: In reference to Dr. Bagshaw's discussion on the incidence of pelvic lymph node involvement in patients with carcinoma of the prostate (2), it might be of interest to say a word about the experience we've had over the past 4 years. We have dissected pelvic lymph nodes in conjunction with ^{125}I implantations in approximately 100 patients with stage B or C carcinoma of the prostate. In about 25% of the patients with stage B carcinoma and in about two thirds of the patients with stage C carcinoma we have found positive pelvic lymph nodes. The point that requires emphasis here is that the first and most common site of lymph node metastases in our experience has been the obturator lymph nodes, and these lymph nodes were those that were not consistently sampled, as I understand it, in Dr. Bagshaw's experience. Furthermore, they are the lymph nodes that would only rarely be shown on the conventional pedal lymphangiograms. This would be a source of "missed" metastasis in the methods of diagnosis that Dr. Bagshaw employed.

D. Coffey: I believe that Dr. Mostofi has given us a very informative presentation (3), particularly when he showed the vast differences in the many types of prostatic cancer we are treating. I'm sure this is common knowledge to all people in the clinical field. In our present clinical studies we are treating patients with advanced disease where we expect very little response. We are using standard cancer chemotherapeutic drugs for which we already know there is at best only a 30%–40% partial remission rate in other responsive tumors. It is therefore important that we compare very carefully those patients who respond with those who do not, with a histologic and pathologic sample versus an historical sample, because from this we may learn our most valuable information and predictions. For example, Drs. Catalona and Scott have treated a patient with 5-fluorouracil [3] who had a remarkable recovery and partial remission although he later relapsed with the disease. I think these are the types of patients that we should analyze very carefully. In addition, I would like to say that what Dr. Merchant said in his presentation (4) was quite accurate. When this program started, as a basic scientist I had serious reservations about the organ site approach. From what I've seen at this workshop I would like to join him in stating that I think this approach has been a tremendous step forward in our understanding and attack on prostatic cancer. Although everything has not been of even quality, we've nevertheless brought many experts from other disciplines into this field, and in this way I hope we will have some future progress.

W. Voigt: With respect to Dr. Mittelman's trials with estramustine phosphate [4] (5), I would like to discuss the following. The patients he selected for study were only those whose tumors had already shown a lack of hormonal responsiveness. Yet, in the design of the alkylating agent some degree of tumor specificity was meant to be conferred by including the estrogen moiety. Wouldn't it have been more appropriate and encouraging to have tried estramustine phosphate in the treatment of patients whose carcinomas were still thought to be responsive to hormonal therapy? I would also like to make a comment about therapy with 5-FU. We have seen at this workshop the variability of response to 5-FU therapy for prostatic cancer (only 20%–25% of the tumors respond). I believe some degree of predictability of this response could be achieved by measuring in tumor samples some key enzymes in the metabolism of 5-FU. I am presently attempting to establish a correlation, in the case of breast cancer, between levels of pyrimidine phosphorylase and thymidine kinase and the response to 5-FU therapy. These enzymes are necessary to metabolize 5-FU to its active form, 5-fluorouridine monophosphate. Our preliminary observations indicate that a correlation does exist. I believe that something like this should be attempted in the case of prostatic therapy with 5-FU in hopes of identifying

[3] 5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8; 5-FU.

[4] Estramustine phosphate: NSC-89199; CAS reg. No. 4891-15-0; estra-1,3,5(10)-triene-3,17β-diol, 3-[bis(2-chloroethyl)carbamate]17-(dihydrogen phosphate); Estracyt.

earlier those patients most likely to benefit from this drug.

A. Mittelman: I think Dr. Nilsson can answer your question in the last slide he showed in his presentation (6). He is treating patients who you may consider hormonally responsive with estramustine phosphate and the results certainly seem encouraging.

J. Grayhack: I have a statement and a question for Dr. Merrin and one for Dr. Bagshaw. My impression is that there is a great deal of literature, Dr. Merrin, on the effect of installation of boiling water, croton oil, and numerous other substances into the prostate. These are said to be effective in the treatment of benign prostatic hyperplasia. I wonder whether the effects which you have noted are really specific or not. Dr. Bagshaw, as I've followed patients who have received radiation therapy to the prostate, it's my impression that many of them have a significant anatomic change in the testis and in the suprapubic area. When I saw the result you achieved in patients with elevated acid phosphatases (as I recall, 15 of 16 or 14 of 15 returned to normal with treatment in the periaortic areas), I wondered whether somehow or other you might be affecting testicular function and whether you have any anatomic or physiologic evidence in this regard.

M. Bagshaw: The change in the suprapubic region which you describe is subcutaneous fibrosis. It can occur when the entire pelvic dose is administered through anterior and posterior parallel opposed fields with cobalt-60 irradiation or X-rays from medium-energy linear accelerators. This can produce great disability with edema of the penis and scrotum and even the lower extremities, but it can be avoided with a modification of the technique. Because we have always used rotation or multiple fields, we have never seen this complication in our patients. It is a devastating sequelae of radiation and should be avoided. Thus, either rotational or at least four-field radiotherapy should be used. The only thing I can say about treatment of the testes is that they should not be included in the radiation fields. The technicians must be careful, particularly when they're treating from the posterior region, because the testes can flop anteriorly and actually be exposed to a full dose of radiation. The dose to the testes from scatter is about 200 rads delivered during the entire course. This should not reduce testosterone production but we have not measured this. Even this low dose can be reduced further to 20 rads by enclosing the testes in an appropriate lead shield 1 cm in thickness. The only physiologic evidence we have that our treatment does not reduce the testosterone levels is that about 60% of our patients remain sexually potent after therapy. In regard to Dr. Whitmore's comment, I think that that is perfectly true. Our surgical exposure is different and our urologists don't dissect deeply enough to get an obturator node all the time. We would like to sample the obturators; however, inasmuch as you're going deeper retroperitoneally into the periprostatic region, I think it's easier for you to pick them up, and that's good information to have.

C. Merrin: Dr. Grayhack, we were certainly aware of the possible necrosis in the prostate by the injection of some foreign material. But I think there are a certain number of facts against this possibility. First, most of the combinations which were used and reported in the literature were caustic. The most effective was a mixture with tannic acid which was used by a British physician practicing in India. He reported successful treatment of benign prostatic hyperplasia by direct injection in the prostate. The croton oil which you mentioned is also very caustic. The bacillus Calmette-Guérin (BCG) per se is not caustic and the volume which is injected in the prostate is minimal (1 cc once a week). This was sufficient to produce necrosis in the two patients. The other thing in favor of direct BCG action in the tumor is the variation of the immune response in these patients and the abundant experimental and clinical material in the literature proving BCG activity.

R. Gibbons: I also have a question for Dr. Bagshaw. This refers to his survival figures where he showed us that approximately 40% of those patients who received radiation therapy as primary treatment for their prostatic cancer were alive at 10 years. My question is how

many of those are alive with no evidence of tumor?

M. Bagshaw: This information was published last year (7). At 10 years, 13 of 28 patients (46%) with tumor limited to the prostate were living without evident disease; only one was alive with cancer. Nine had died without cancer and five had died with cancer. Also at 10 years, six of 23 patients (26%) with extracapsular extension were living without evident neoplasm and one was alive with cancer. Four had died without cancer and 12 had died with cancer.

M. Pollard: No doubt Dr. Merrin knows of other immunopotentiating agents that have been used recently, namely *Corynebacterium parvum* and *Corynebacterium granulosum*. I wonder if he knows of anyone who is using or intending to use these as a substitute for BCG.

C. Merrin: Yes. I was thinking of using a different bacterial agent but the main problem was the organ specificity for the given bacterial agent; it seemed that most of our BCG injected in the prostate migrated elsewhere. We were able to find granulomas in the lungs of our patients, and there was no evidence of granuloma formation in the prostate. I thought of using bacteria which are very specific to the prostate. I even thought of using gonococcus, but after analysis I found it could not be very helpful.

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ANNOUNCEMENTS

Annual Meeting of the American Association for Cancer Research, Inc.

San Diego, California, May 8–10, 1975

The scientific sessions of the 66th Annual Meeting of the American Association for Cancer Research, Inc. will be held at the Town and Country Hotel, San Diego, California, from 8:30 AM, Thursday, May 8, until 5:00 PM, Saturday, May 10, 1975.

Registration will be held on the afternoon and evening of May 7 and during the meeting. Nonmembers may obtain programs and purchase abstracts of the papers at the time of registration. By action of the Board of Directors, a registration fee of $15.00 will be charged each member and nonmember attending the scientific sessions; a special rate of $5.00 will be charged predoctoral students who present a statement signed by the registrar, dean, or department head of their university or college confirming their status. Admission to the scientific sessions will be restricted to properly registered persons.

Abstracts that have been submitted in conformity with regulations and considered of acceptable quality will be published in the March 1975 *Proceedings of the American Association for Cancer Research* which will be mailed by the publisher to all paid-up subscribers to the journal *Cancer Research*.

For further information write to: Hugh J. Creech, Secretary-Treasurer, American Association for Cancer Research, The Institute for Cancer Research, Fox Chase, Philadelphia, Pa 19111.

Examination of the American Board of Internal Medicine in the Subspecialty Area of Medical Oncology

The examination of the American Board of Internal Medicine in the Subspecialty Area of Medical Oncology will be administered on Tuesday, October 21, 1975. Registration begins January 1 and ends March 15, 1975. For information and application forms please write to: American Board of Internal Medicine, 3930 Chestnut Street, Philadelphia, Pa. 19104.

Symposium on Breast Cancer

Teheran, Iran, April 14–16, 1975

This symposium will be organized by the Taj Pahlavi Cancer Institut of Teheran University in collaboration with the World Health Organization, Eastern Mediterranean Region, Institut Gustav-Roussy, Villejuif, France.

All correspondence should be addressed to: A. Mojtabai, MD, Taj Pahlavi Cancer Institut, P. O. Box 14/1154, Teheran, Iran.

CORRECTION

Page 862 of the November/December 1974 issue of *Cancer Chemotherapy Reports* (Vol. 58, No. 6) contains an error. The last sentence of the first full paragraph should read as follows: "Tolerable toxicity reported in a phase I trial by Cohen and Mittelman was experienced at a dose of 4 mg/m^2 (2)."

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